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U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

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Mycotoxins	Clinical Trials	Volunteers,	~~RAD I~~
Marine Toxins	Chemotherapy	Immunology,	Expert Systems
Rapid Diagnosis	Pharmacology	Entomology	Prophylaxis (55)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A report of progress on the research program of the U. S. Army Medical Research Institute of Infectious Diseases on Medical Defense Against Biological Agents (U) for fiscal year 1985 is presented.

Section 19 - KEYWORDS - CONTINUED FROM PAGE iii

Low Molecular Weight Toxins
Small Molecular Weight Toxins
Recombinant DNA Technology
Rickettsial Diseases
Rapid Identification
Biological Warfare Defense

EDITOR'S NOTE

This FY 1985 Annual Progress Report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, conducted on Medical Defense Against Biological Warfare Agents (U) under Projects 3M161102BS12, 3M162770A870, 3M263763D807, 3M463750D809, 3M162770A871, 3S464758D847, and In-House Laboratory Independent Research, Project 3A161101A91C.

In conducting the research described in this report, the investigators adhered to the: a. "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care, and b. "Guidelines for Research Involving Recombinant DNA Molecules" published by the Department of Health and Human Services, National Institutes of Health in the Federal Register May 7, 1986 and erratum thereto.

FOREWORD FY 85

I. USAMRIID's MISSION

USAMRIID develops strategies, products, information, procedures and training for medical defense against biological warfare agents and naturally occurring infectious agents of military importance that require special containment.

The Army has triservice responsibility for medical defense against biological warfare as referenced in DoD Directive 5160.5, "Responsibilities for Research, Development, Test and Evaluation (RDT&E) on Chemical Weapons and Chemical and Biological Defense," 30 March 1976; and the joint service agreement on joint service planning and execution of chemical/biological defense systems Research, Development, Test, and Evaluation (RDT&E), 5 July 1984.

II. DISSEMINATION OF INFORMATION

All research conducted at USAMRIID is unclassified. Results of research are published in peer-reviewed scientific literature as well as in annual reports. Oral and poster session presentations are given at meetings of numerous national and international scientific societies. Results of value to organizations outside the U.S. Department of Defense are shared willingly, in the hope that such sharing will result in additional information on the validity of scientific results or the efficacy of new products, such as vaccines, other biologicals, or drugs. Numerous domestic and international collaborations exist. USAMRIID established an Office of Technology Transfer in FY 82 in order to fully implement the Stevenson-Wydler Innovation Act of 1980. USAMRIID prints a cumulative bibliography of published articles, which may be obtained by a request to the Editor, USAMRIID, Fort Detrick, Maryland 21701-5011.

III. INSTITUTE HIGHLIGHTS FOR FY 1985

The research programs of USAMRIID are designed to provide a medical defense to US Forces against biological warfare (BW) by potential adversaries. Fiscal year 1985 reflected a new awareness of their potential threat and a series of reviews were conducted which resulted in an expanded program, both in funding, manpower, and facilities for the five-year period FY 1986-1990.

As a result of increased resources, USAMRIID research and development programs are undergoing major realignment, shifts in emphasis, and most importantly, are being significantly expanded. Prior to the review, the medical BW defensive program concentrated largely on conventional agents and toxins, which included mycotoxins and botulinum neurotoxins. The expanded program will include all of the above plus new priority research efforts on recently recognized threat agents. In addition, the program will concentrate on generic approaches to defense where therapy for a group of potential threat agents will be considered of highest priority. Research programs have been structured to accelerate development on current experimental vaccines, toxoids, and drugs in order to get them fielded, licensed, and stockpiled, thus improving the readiness posture of all forces. Before licensure, each product requires Phase III testing in approximately 4,000 volunteers who must come from many elements of the Army and perhaps from the other services. Policy to cover product usage and stockpiling is in the process of being formulated by the Surgeon General.

One of the first actions following the review was to restructure the Office of the Commander in order to better manage the expanding and realigned programs. Two new offices were created: Deputy for Research and Deputy for Product Development. A contract management office, staffed with three people, was established under Deputy for Research because about 60 percent of the expansion will occur in the extramural contract program. Another action by USAMRIID management to meet new challenges posed by the review was to hold an intensive review and analysis of the entire program.

There is a growing public awareness of the Soviet Union's ability to exploit genetic engineering in order to create new biothreats that can be used in biological warfare. Articles in the Wall Street Journal, The Washington Post, The New York Times, and other leading newspapers and magazines have described the vast potential of these new biothreats. Television has played an even greater role in educating the public. NBC, BBC, and "Sixty Minutes" of CBS have either produced or have scheduled programs on BW with USAMRIID as a centerpiece. Institute policy has been to allow the press access to laboratories and staff and to be responsive to their requests. Unfortunately, the media have emphasized the sensational, blurred distinctions between offensive and defensive research, and left the impression that somehow USAMRIID conducts secret offensive BW research. Senior management spends an inordinate amount of time and energy trying to neutralize these false images of a program that is unclassified and dedicated entirely to medical defense.

FY 1985 constituted a rejuvenation of the testing of new products in the military research volunteer subject (MRVS) in USAMRIID's unique experimental P-3 (Biosafety levels) containment ward. Although these young and highly motivated soldiers are assigned to USAMRIID, they provide an invaluable resource for the entire U.S. Army Medical Research and Development Command (USAMRDC). Important studies conducted in volunteers during FY 85 include the following: USAMRIID performed four Rift Valley fever vaccine studies, one Western equine encephalitis vaccine study, and a key study testing the efficacy of the antiviral drug ribavirin to control sandfly fever infection. In cooperation with the Walter Reed Army Medical Institute of Research (WRAIR), USAMRIID performed two antimalarial drug studies, and one *Shigella* vaccine study in its experimental P-3 containment ward. In cooperation with the U.S. Army Medical Research Institute of Chemical Defense, USAMRIID performed two tests with pyridostigmine, an anti-nerve-agent drug, also in USAMRIID's containment ward. In cooperation with USAMRIID, the U.S. Army Research Institute of Environmental Medicine performed one test concerned with high altitude environments. About 90 percent of the 83 MRVS currently assigned to USAMRIID have participated in at least one study, while some have volunteered for two and occasionally three studies.

The importance of the MRVS and the experimental containment ward (containment minimizes inadvertent infections from casual contact) cannot be overstated in generating the necessary data to bring an experimental product successfully through the transition from laboratory use to field use. Two examples will illustrate this point.

1. A major advancement in developing an antiviral agent was made with the evaluation of the drug ribavirin for the prevention of Sandfly fever virus infection in MRVS. In this phase II clinical study, volunteers received ribavirin, 1200 mg/day orally for eight days, or placebo beginning one day prior to inoculation with virus. Illness was prevented in all individuals receiving ribavirin, while most placebo-treated control subjects became ill, demonstrating a typical febrile and leukopenic response to the virus. Of even greater significance, however, was the observation that five of six ribavirin-treated subjects seroconverted after virus inoculation. Hence, this study demonstrated that ribavirin, while protecting against disease, does not prevent the development of antibodies and the immune state in treated individuals. These results provided the necessary information on which to proceed with implementing a study in Argentina concerned with treating Argentine hemorrhagic fever patients with ribavirin.

2. In collaboration with investigators at WRAIR and the National Institutes of Health, the medical staff of USAMRIID conducted clinical studies leading to the selection of two antimalarial drugs. Both enpirolone and halofantrine were found to be effective against multi-drug-resistant strains of malaria from Southeast Asia. Effective treatment of malaria is a major goal of military medicine, since malaria has constituted a serious medical problem in every conflict in which U.S. Forces have been involved. This therapy will provide an interim control while other efforts directed to an effective vaccine can be completed.

Another event which has had positive impact on Institute performance and visibility was its sponsorship of an "International Workshop on the Molecular Biology of Flaviviruses" held 29 November to 1 December 1984. About 140 scientists, including 10 International experts, participated in this meeting. The Dengue Steering Committee Scientific Advisory Group of the World Health Organization attended the workshop and used this information exchange to plan strategy for extramural support of flavivirus research. The objectives of this workshop were to examine the current status of the molecular virology of these agents and develop concepts for future flavivirus vaccine development. The USAMRDC flavivirus research program was modified as a direct result of this scientific exchange and numerous collaborative research projects were initiated.

USAMRIID has organized its military personnel into 32 biologic/toxin rapid-reaction teams. These teams are composed of specialists and are prepared for rapid deployment on a global basis to support U.S. Forces and their allies in situations where biological/toxin contamination has occurred or is suspected of having occurred. On-site assessment is essential to establishing whether or not BW agents have been used.

USAMRIID continues to maintain a dynamic National Academy of Science/National Research Council postdoctoral training program. The program started a few years ago with just nine authorizations and doubled to 18 authorizations last year. The program was staffed with an average of 11 scientists during FY 1985. The yearly independent audit of USAMRIID scientists and research programs by the NAS indicates that the high standards and quality of the research environment in this Institute continue unabated.

USAMRIID is pleased to announce that one of its scientists, Dr. John W. Huggins, was presented the prestigious "Outstanding Employee Professional Award", by the Baltimore Federal Executive Board on 26 April 1985 for his scientific achievements. This award is given annually to the outstanding individual from the 70,000 civilian employees from the various federal agencies in the Baltimore metropolitan area. Dr. Huggins developed an animal model for Korean hemorrhagic fever, then used this model to show that the new antiviral drug, ribavirin, is effective in treating this dangerous and militarily relevant disease. This research has led to the establishment of a clinical trial of ribavirin in the People's Republic of China.

The Institute is also pleased to announce that the Department of the Army has awarded its "Research and Development Achievement Award" to Lieutenant Colonel James W. LeDuc, Chief, Department of Epidemiology, Disease Assessment Division. The award was granted in recognition of his contribution to the understanding of the viruses causing hemorrhagic fever with renal syndrome. This work revealed for the first time the existence of an important new group of viruses, defined its broad global distribution, and developed the methods to analyze them successfully. The award is given to Army scientists or engineers whose work is responsible for a significant scientific or engineering achievement that was made or culminated during the preceding year. Similarly, USAMRIID was pleased to receive the "Award for Excellence in 1984" in laboratory-of-the-year competition, as outlined in AR672-305.

Three research accomplishments were particularly outstanding and require special mention. First, a major discovery was made in the treatment of highly lethal saxitoxin intoxication. Previous to these observations, the only effective therapy against saxitoxin was the use of supportive therapy; for example, a mechanical respirator. A polyclonal antibody was developed which will provide passive immune protection against saxitoxin challenge. Treatment with the antibody is effective when given intravenously after a systemic exposure to the toxin. Furthermore, we demonstrated that in instrumented rats, the antibody was effective, even when the animals had respiratory arrest following saxitoxin intoxication. The second area of outstanding research comes from the ILIR program and is concerned with the cloning of cobra toxin genes in order to study the feasibility of developing safe and effective toxoids against the toxic components of the venom. In just nine months, 130 clones have been produced, the three major toxins have been purified to homogeneity, and polyclonal antibodies have been prepared in rabbit serum and affinity-purified to remove casual contamination with E. coli antibodies. The 130 clones are now being screened by means of DNA probes to locate and select the gene responsible for each toxin. The program is progressing much faster than anticipated. These results clearly demonstrate the ease by which an adversary can now produce sufficient amounts of natural toxin for BW weaponization; whereas, prior to recombinant DNA biotechnology, this was impossible. The third area of outstanding research concerned a major advancement in developing a second generation of safer and more effective vaccines through the use of biotechnology. The feasibility of this approach was strengthened when Rift Valley fever virus (RVFV) genes, G-1 and G-2 glycoproteins, were inserted in a vaccine carrier, vaccinia virus. Mice that received this recombinant virus vaccine were protected against conventional routes of challenge with virulent RVFV. The recombinant vaccine perhaps could be further improved by inserting only the G-2 gene. More recent information indicates that it is the G-2, not the G-1, glycoprotein that is responsible for immunity.

USAMRIID has been productive both at the laboratory bench and in its publication of research results. New vaccines, new treatment modalities, and new diagnostic assays for unusual diseases and toxins provide concrete evidence of this productivity. Moreover, for the second consecutive year, manuscripts published in recognized, scientifically refereed journals are at an all time high. All of this information, although generated for dangerous agents with BW potential, has broad application to both military and civilian medicine. It may be concluded quite reasonably that USAMRIID has established itself as a center of excellence which fulfills a unique medical need by performing research on high hazard agents that cannot be studied as extensively anywhere else in the free world.

Questions or comments about this report are welcomed and may be addressed to:

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Fort Detrick
Frederick, Maryland 21701-5011

AUTHOR INDEX

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b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Middlebrook, J L
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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Microbial Toxins; (U) Biochemistry; (U) Therapy; (U) Lab Animals; (U) Guinea Pigs; (U) Ram I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective of this work unit is to provide the research base for the development of protective modalities from toxins. Recent advances in molecular genetics have demonstrated that many toxins can be cloned and produced in mass quantities. We are studying several toxins of high potential for BW use with these new techniques.

24. (U) Our approach is to study any and all aspects of the toxin including detection, genetics synthesis, elaboration, structure, composition, pharmacology, mechanism of action, pathogenesis, and sensitivity to drugs. We seek to develop novel means of protection from botulinum and marine toxins as well as mycotoxin. Some of these are synthetic vaccines, CRM-based vaccines, toxin-blocking drugs or toxin-reversing drugs.

25. (U) 8410-8509 Ultrastructure studies of T-2-treated liver cells showed changes in only two organelles, endoplasmic reticulum and mitochondria. Studies with two other cell lines and T-2 indicated that energy depletion would result in a higher level of T-2-cell association due to increased uptake, decreased export, or both processes. Polyclonal antibody was raised against crotoxin and to its subunits. This antibody was then used to develop ELISA's for rattlesnake neurotoxins. The cross-reactivity to other rattlesnake neurotoxins was high and the antisera neutralized crotoxin and its basic subunit. Improved purification schemes were developed for several botulinum toxin serotypes. This toxin was shown to inhibit guanylate cyclase from nerve cells and synaptosomes; the poisoning of synaptosomes could be partially reversed by cGMP. Phosphoinositide turnover was also affected by botulinum toxin. Several viral proteins of interest were partially sequenced.

BODY OF REPORT

PROJECT NO. 3M161102BS12: Science Base/Medical Defense Against BW

WORK UNIT NO. S12-AA-001: Characterization of Microbial Toxins of BW Importance

PRINCIPAL INVESTIGATOR: John L. Middlebrook, Ph.D.

ASSOCIATE INVESTIGATORS: Martin Crumrine, MAJ, Ph.D.
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Bruce Meade, Ph.D.
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Leonard Smith, Ph.D.
Lynn Trusal, MAJ, Ph.D.

Background:

This work unit has undergone a major transformation during the past year. With the emergence of molecular genetics has come an increasing anxiety over the possible development of new and powerful toxin-based biological weapons by other nations. One area of special concern is peptide or protein toxins. The structural genes coding for these toxins could potentially be cloned and, in the right expression system, the toxins produced in unprecedented quantities. Indeed, there is already a report in the literature describing the successful cloning of erabutoxin, a very potent neurotoxin from the sea snake, *Laticauda semifasciata* (1). In response to this new threat, the research encompassed by this work unit has expanded to include several toxins of nonmicrobial origin. At present, projects are underway with both pre- and post-synaptic neurotoxins of bacterial and snake origin, protein synthesis inhibitory toxins of microbial and plant origin, and small molecular weight toxins from fungi.

The goal of this work unit is to develop and evaluate possible toxin-protective modalities. We have taken a comprehensive approach to the problem and are proceeding along two general lines of investigation: the development of vaccines and the development of chemotherapeutic agents. To these ends, we study all the aspects of toxins including genetics, synthesis, structure, pharmacology, immunology, and mechanism of action.

Summary:

Ultrastructure studies of T-2-treated liver cells showed changes in only two organelles, endoplasmic reticulum and mitochondria. Studies with two other cell lines and T-2 indicated that energy depletion would result in a higher level of T-2 cell association due to increased uptake, decreased export, or both processes. Polyclonal antibody was raised against crotoxin and to its subunits. This antibody was then used to develop ELISAs for rattlesnake neurotoxins. The cross-reactivity to other rattlesnake neurotoxins was high and the antisera neutralized crotoxin and its basic subunit. Improved purification schemes were developed for several botulinum toxin serotypes. This toxin was shown to inhibit guanylate cyclase from nerve cells and synaptosomes; the poisoning of synaptosomes could be partially reversed by cGMP. Phosphoinositide turnover was also affected by botulinum toxin. Several viral proteins of interest were partially sequenced.

Progress:

T-2 Mycotoxin Because other research at this Institute has shown that the liver is a target organ for T-2, and because it was desirable to study the toxicity of T-2 in a primary cell line, the biochemical and morphological effects of T-2 on hepatocytes were evaluated. Cultured rat hepatocytes were treated with several doses of T-2 for either 1 or 12 h and with or without a 12-h recovery period. Inhibition of protein synthesis and release of lactate dehydrogenase were measured and correlated with ultrastructural changes, as assessed by transmission electron microscopy. Results indicated that, at a dose of 0.01 μg/ml, protein synthesis was inhibited 75% within 1 h, but recovered to near control levels with or without the continual presence of toxin. At the higher toxin dose (1.0 μg/ml), hepatocytes were able to recover from a 1-h, but not a 12-h exposure. Cell damage, as assessed by release of lactate dehydrogenase, lagged behind inhibition of protein synthesis. Only at a T-2 concentration of 1.0 μg/ml for 12 h followed by a 12-h recovery period, was release of lactate dehydrogenase significantly increased over control values. Under the same parameters, protein synthesis was inhibited 94%. The ultrastructural appearance of the cell membrane, nucleus, lysosomes, peroxisomes, and smooth endoplasmic reticulum remained unchanged. The two organelles which appeared altered by the T-2 exposure were the rough endoplasmic reticulum and the mitochondria. Endoplasmic changes were limited to degranulation of attached ribosomes without dilation of the cisternae. Alterations were seen as early as 1 h at a T-2 dose of 0.01 μg/ml. After a dose of 1.0 μg/ml for 12 h, some mitochondria displayed one or more non-membrane-bound translucent foci, some of which contained electron-dense cores.

A second study was conducted to evaluate energy-dependent mechanisms that may affect uptake of T-2 toxin at the cellular level. Chinese hamster ovary (CHO) and African green monkey kidney (Vero) cells were seeded into 24-well plates. Cells were preincubated for 1 h at 37°C in media with (experimentals) or without (controls) sodium fluoride (NaF) at 5 mM. All wells then received tritium [^{3}H]-labeled T-2 at a concentration of 0.001 or 0.01 μg/ml. After a rinse, cells were digested (0.01 N NaOH) and counted in a liquid scintillation counter. Cell-associated T-2 was expressed as molecules/cell, based on the specific activity of [^{3}H]T-2 and replicate cell counts. Statistical analysis of individual points along the curve demonstrated that NaF significantly ($P < 0.05$) increased cell-associated toxin at most time points. This was true regardless of cell type or toxin concentration. Vero cells accumulated significantly ($P < 0.05$) more T-2 than did CHO cells in the presence of NaF and at the higher dose of T-2 in the absence of NaF. The reason for differences in uptake between Vero and CHO cells is unknown, but may reflect cell type-specific factors. It is known that T-2 binds intracellularly. Furthermore, NaF is known to inhibit cellular events dependent on ATP produced by glycolysis. Therefore, data from this study suggest that inhibition of glycolysis leads to an increased cellular uptake of T-2, decreased cellular export of T-2, or a combination of both events.

Crotoxin Crotoxin is the major toxic protein found in the venom of the South American rattlesnake (*Crotalus d. terrificus*). It consists of a moderately toxic basic phospholipase A_2 (i.v. $LD_{50} \simeq 0.5$ μg/gm in mice) and a non-toxic, acidic subunit composed of three small proteins. The non-covalent association of the two subunits enhances the toxicity ($LD_{50} \simeq 0.05$ μg/gm) and promotes irreversible blockage of neuromuscular transmission. Details of this blockage are poorly understood. We have been working to gain a better understanding of the mechanism of action of crotoxin and also to obtain a clearer picture of crotoxin's relationship with other rattlesnake neurotoxins. Our long-term goal is to clone the crotoxin gene for future site-specific mutagenesis studies.

The sequences of the three acidic subunit chains of crotoxin have been determined. Primary structural data suggest that both the acidic and basic subunits have been derived independently from a non-toxic, hemodimeric, crotalid phospholipase A_2. When compared with sequences of other phospholipase A_2s, the acidic subunit lacks a 22-residue, amino-terminal fragment and also two additional internal segments. It retains His-47, Asp-48, and Asp-91, all found conserved in phospholipase A_2s and believed involved in Ca^{++} binding and catalysis. Yet, crotoxin displays no phospholipase activity.

Polyclonal antibodies have been prepared in rabbits against crotoxin, the acidic and basic subunits of crotoxin, and the principal toxin in venoms from *C. s. scutulatus* and *C. v. concolor*. In collaboration with Major Martin Crumrine, we have developed an ELISA for rattlesnake neurotoxins that uses horseradish peroxidase. High sensitivities permit routine coating of wells with 100 ng of antigen and antibody serum dilutions of 250,00 to 1 million.

Antiserum raised against intact crotoxin showed strong cross-reactivity with purified toxins from *C. s. scutulatus*, *C. v. concolor*, *C. vegrandis*, and the basic subunit of crotoxin. Phospholipase A_2 from *C. adamanteus* and *C. atrox* cross-react to a lesser extent, but better than either β-bungarotoxin or the acidic subunit of crotoxin. We see no reaction with the phospholipase A_2s from honey bees or *Naja naja atra* or bovine neurophysin.

Ouchterlony assays with crotoxin antibody show single lines of identity between crotoxin and the three purified toxins from *C. scutulatus*, *concolor* and *vegrandis* venoms.

Rabbit antisera against intact crotoxin and each of the subunits have been examined for their ability to neutralize intact crotoxin in mice. In experiments where the toxin and serum were pre-mixed, one ml of intact toxin antibody neutralized 340 LD_{50}s of crotoxin, and basic subunit antibody neutralized twice that amount (approximately 640 LD_{50}s). Serum containing antibodies to the acidic subunit neutralized less than 50 LD_{50}s. Preliminary experiments suggest that antisera given after crotoxin administration to mice must be done so within 15 min or less to be effective.

Preparations for cloning the crotoxin gene are well underway. We obtained three South American rattlesnakes (*C. vegrandis*) and have made arrangements for their milking, venom gland removal, and mRNA/DNA isolation at the University of Wyoming. We have purified the crotoxin-specific IgG fraction from rabbit antiserum by affinity chromatography on a crotoxin-Sepharose column and are setting up the methodology to screen for *E. coli* clones synthesizing crotoxin. Several synthetic deoxyoligonucleotides have been chemically synthesized and purified and will serve as probes in this cloning work. Much of the biotechnology developed with the *N. n. atra* cloning work will be directly applicable to this problem.

In an effort to gain insights into crotoxin's mechanism of action, we have initiated several approaches. First, we optimized conditions for the chemical cross-linking of crotoxin. This material should permit us to address the question of whether subunit dissociation is essential for toxicity. Second, polyclonal IgG from rabbits immunized against crotoxin has been purified using DEAE Affi-Gel Blue. This fraction was conjugated with rhodamine isothiocyanate and is being used to examine crotoxin distribution on NG 108-15 nerve cells. Preliminary experiments demonstrated quite intense staining of cells incubated with crotoxin. Finally, we have carried out a number of preliminary studies using synaptosomes and tissue culture cells (Vero, PC-12, NG 108-15) to examine the effects of crotoxin and its subunits on choline uptake and acetyl choline release. It seems clear that one of the major effects of crotoxin is its inhibition of choline uptake. These studies are continuing.

Cobra Toxins Cloning of the genes for three toxins from *N. n atra*, the Formosan cobra, and evaluation of the protein products are underway. Technical details of the work are filed under work unit 91C-00-140.

Botulinum Neurotoxin Botulinum toxin (Botx) is a generic term describing seven serologically distinct toxin proteins isolated from *Clostridium botulinum*. Another potent neurotoxin having features resembling botulinum toxin is tetanus toxin (Ttx), isolated from *C. tetani*. Unlike the Botx, Ttx is found in only one serological form. Although these eight toxins are antigenically distinct, they have three important features in common: (i) they are synthesized as single polypeptide chains by the same genus of bacteria, *Clostridium*; (ii) they have similar molecular weights and protein structures, and (iii) they block neurotransmitter release from the presynaptic nerve ending. Tetanus toxin is known to have two apparently different actions, central and peripheral, while botulinum toxin affects only the peripheral cholinergic nerve endings. When Botx or Ttx is injected intramuscularly, the clinical symptoms and time course to poisoning are very different. However, it was recently shown that i.v. injection of Ttx produces a very rapid, flaccid paralysis indistinguishable from the symptoms of Botx poisoning (2). The molecular mechanism by which these toxins cause the inhibition of acetylcholine release is not known. However, theophylline, an inhibitor of phosphodiesterase activity, is known to antagonize the toxic effects of Botx and Ttx (3). In addition, when rabbit sphincter pupillae muscles are paralyzed with Ttx, cyclic guanosine monophosphate (cGMP) injections temporarily reverse the paralysis of these muscles (4). These observations provide indirect evidence that cGMP may be implicated in the mode of action of Ttx and stimulated us to investigate the effects of both Botx and Ttx on cyclic nucleotides.

The effects of botulinum and tetanus toxins on the cyclic nucleotide levels were assessed in synaptosomal extracts as well as extracts from the nerve cell lines NG108-15 and PC-12 and the monkey kidney cell line, Vero. The neural cell lines were prepared in both the differentiated and undifferentiated states. Cell extracts were prepared by hypotonic lysis in 1mM Tris-Cl. A P_2 fraction of synaptosomes was prepared from the cerebral hemisphere of guinea pigs and synaptosomal extracts were prepared by homogenization of the synaptosomes in 1mM Tris-Cl. Synaptosomal or cell extracts were preincubated with toxin ($10^6 LD_{50}$/assay) for 30 min at 37°C, followed by the addition of cyclase substrates. Reactions were then continued for 30 min at 37°C and stopped by heating to 90°C for 2 min. Cyclic nucleotides were quantitated by radioimmunoassay (New England Nuclear RIA kit for cAMP and cGMP). We found that Botx and Ttx inhibited the guanylate cyclase activity found in both guinea pig synaptosomes and NG 108-15 cells. In lysed synaptosomes or cells, inhibition was complete in 30-60 min. The magnitude of this inhibition, for both soluble and particulate forms of the enzyme, was as high as 60%. The effect was observed with three different Botx serotypes: A, C, and D. In paired experiments, no effect was observed on adenylate cyclase; neither toxin exhibited phosphodiesterase or phosphatase activities. Since cGMP is important for neurotransmitter release, it is possible that this biochemical action of Botx and Ttx explains their toxicities, but further experiments are required to establish this connection.

One attempt along these lines was to ask whether the addition of exogenous cGMP would reverse the inhibition of acetylcholine release caused by the toxins. Synaptosomes were prepared in non-depolarizing buffer (2.5 mM K^+ and 137 mM Na^+) and incubated with 10^6 mouse LD_{50}/ml of botulinum toxin with and without nucleotides. The synaptosomes were pulsed during the final 20 min of incubation at 37°C with [^{3}H]choline chloride. Following the pulse, synaptosomes were rinsed and resuspended in non-depolarizing buffer. Aliquots were added to either non-depolarizing or depolarizing buffer (25 mM K^+ and 114 mM Na^+) at 37°C for 20 min to stimulate the release of acetylcholine. Following the release interval, synaptosomes were pelleted and the supernatants were quantitatively analyzed for acetylcholine content. In our experiments, we are able to inhibit the release of acetylcholine in guinea pig synaptosomes up to 70% by using 10^6 mouse LD_{50}/ml of botulinum A or D toxin. If the inhibition assay was performed in the presence of 1 mM cGMP or cAMP, between 50-100% of the acetylcholine release potential was restored. It must be pointed out, however, that cGMP, as well as cAMP, induced a basal (nondepolarizing buffer) increase in the release of acetylcholine as well as an increase in K^+-evoked release from synaptosomes not treated with Botx toxin. This was in contrast to results obtained in the presence of cAMP where there seemed to be an enhancement in the Botx-induced inhibition of acetylcholine release. Obviously, the situation is complex and further studies will be required to determine whether the nucleotide effects are specific to the action of the toxin. Control experiments showed that the cyclic nucleotides were very rapidly hydrolyzed by phosphatases from the synaptosomal extract. However, when nucleosides are incubated with the synaptosomes, little or no effect is observed upon acetylcholine release, suggesting that the effects seen with the cyclic nucleotides result from the action of these compounds prior to their hydrolysis. Experiments will be performed with non-hydrolyzable cyclic nucleotide derivatives to see if one can prolong the increased release of acetylcholine in the presence of Botx.

A second area of interest has been the effect of toxins on the phosphoinositide turnover in membranes. Phosphoinositides are minor phospholipids (~5% of phospholipid) that turnover much more rapidly than other membrane lipids. A wide variety of neurotransmitters, hormones, growth factors, and many other biologically active substances are known to provoke the turnover of phosphotidylinositol (PI) in their target tissues (5). When stimulated, most of these receptors immediately mobilize Ca^{++}, often release arachidonate, and increase the concentration of cGMP but not cAMP. This PI turnover appears to be a sign for the transmembrane control of cellular functions and proliferation through activation of a novel protein kinase C. In many tissues, both cAMP and cGMP block the receptor-linked phospholipid degradation and thereby counteract this signal translation. As stated above, receptors that mediate PI responses are also capable of calcium gating (6). This observation, plus the fact that the PI response is calcium-independent (7) and that phosphotidic acid (a PI metabolite) can act as a calcium ionophore (8), has led to the hypothesis that the PI response gives rise to calcium gating (9). Because Ca^{++} gating is important to release of acetylcholine (10), we studied the effect of Botx and Ttx on PI turnover in guinea pig synaptosomes. Our initial investigation focused on membrane uptake of [^{3}H]myoinositol, which is a measure of the phosphoinositide turnover.

Synaptosomes were prepared as described above and preincubated with or without toxin (10^6 mouse LD_{50}/assay) for 2 h at 4°C followed by incubation with ^3H-myoinositol for 20-or 40-min intervals at 37°C. Results showed that, after 20 min, Botx or Ttx caused an increase in the uptake of [^3H]-inositol, while after 40 min, a decrease in [^3H]-inositol uptake was observed. It should be pointed out that if one examines acetylcholine release by synaptosomes treated with botulinum A toxin, inhibition is observed only at the 40-min incubation period, not at the earlier time. The increase in PI turnover during the first 20 min at 37°C may represent the initial stages of binding or internalization of the toxin, and the 40-min period presumably represents the poisoning action of the toxin in which PI turnover is inhibited, Ca^{++} channels are closed, and acetylcholine is not released. The effect the toxin has on the level of cGMP may be a secondary effect due to the inhibition of PI turnover. Further experiments are underway to clarify the effect that toxin has on PI turnover and its relationship to inhibiting the release of acetylcholine.

An improved method for the purification of type E botulinum neurotoxin has been achieved. With reference to published methods, the new scheme eliminates pre-concentration steps, size exclusion chromatography, and two cation exchange chromatography steps. Further, protamine sulfate precipitation of nucleic acids was substituted for the ribonuclease treatment. This eliminates a 5-h incubation at 30°C (which sometimes leads to the appearance of proteolytically modified forms of the toxin), and can be completed in one day instead of two. The procedure was developed so that it could be performed with the Pharmacia FPLC system, or with a normal open-column system. At this point, approximately 30 mg of type E have been purified and stored.

Procedures for purifying type B neurotoxin were also explored. Initial attempts used the FPLC system and small amounts of semi-crude toxin. A simple method for obtaining electrophoretically pure toxin was achieved. Scale-up was achieved by working with two preparations of semi-crude type B (these had each been taken through a single DE52 step, starting with the crude extract). A rapid and efficient procedure was developed, quite similar to that for type E. Again, the scheme is adaptable to either the FPLC or open column systems. We now have about 35 mg of purified type B.

Finally, a small amount of semi-crude type A neurotoxin was purified by using the FPLC system. The results suggest that larger amounts may be obtained by using procedures similar to those for E and B. We are hopeful that a majority of the seven serotypes will lend themselves to purification by our scheme, disproving the literature claims that each requires different and time-consuming techniques.

Protein sequencing work Two preparations of G-1 protein and one of G-2 protein from Punta Toro virus were received for amino acid sequence analyses from Dr. Jonathan Smith, Virology Division, USAMRIID. The samples contained relatively small amounts of protein contaminated with detergents. This led to a low signal-to-noise ratio in the detection system. Further, our Beckman 890C sequencer is obsolete and not capable of efficiently analyzing low-level samples. Nonetheless, 16 of the first 20 amino acids in the G-1 protein were identified, as were 6 of the first 12 in G-2. These results allowed the determination of the order of these proteins within the viral genome and the starting points of their nucleotide sequences. A sample of a 14K-polypeptide from Punta Toro virus was also sent by Dr. Smith for analysis, but the amount was so low that no results could be obtained.

Dr. J. Dalrymple submitted a preparation of E-1 protein, and one of E-2 protein (both from Chikungunya virus) for sequence determination. The first 25 residues of E-1 were identified, while 22 of the first 26 of E-2 were identified.

Amino acid sequence determinations were also made for samples sent by LTC Ray Chung, Dr. Bob Seid, and Dr. Wendell Zollinger of WRAIR. Samples included a gonococcal pilin protein and a cyanogen bromide fragment derived from this protein. Forty-six of the first 50 residues in the former polypedtide and the first 44 residues of the latter were identified. The third sample, a preparation of 2F-1 antigen from *Neisseria meningitides*, contained insufficient material for successful analysis.

Amino acid sequence analyses were done to confirm both the purity and identity of several snake toxins. Because of the complexity of most venoms from which the toxins were purified, it is no trivial matter to be certain that one has the correct toxin. In most cases, the derived sequences agreed completely with those published in the literature. In one instance, the initial peptide mapping proved that a company had provided us with the wrong toxin.

PRESENTATIONS:

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2. **Kaiser, I. I., S. D. Aird, W. Kruggel, and R. V. Lewis.** Amino acid sequence studies on the acidic subunit proteins of crotoxin. Presented at 8th World Congress on Animal, Plant, and Microbial Toxins. Newcastle-upon-Tyne, England. August 11-16, 1985.

3. **Leppla, S. H., D. L. Robertson, S. L. Welkos, L. A. Smith, and M. H. Vodkin.** Cloning and analysis of genes for anthrax toxin components. Presented at 2nd European Workshop on Bacterial Toxins, Belgium. June 30-July 4, 1985.

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5. **Smith, L. A., and J. L. Middlebrook.** Botulinum and tetanus neurotoxins inhibit guanylate cyclase activity in synaptosomes and cultured nerve cells. Presented at 8th World Congress on Animal, Plant, and Microbial Toxins, Newcastle-upon-Tyne, England, August 11-16, 1985.

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Table 1

Reactions of various proteins to antiserum raised against the acidic and basic subunit of crotoxin, Mojave toxin, and concolor toxin.

	ACIDIC	BASIC	CROTOXIN	CONCOLOR	MOJAVE
Acidic subunit	++	++	++	++	++
Basic subunit	++	++++	++++	++++	++++
Crotoxin	+++	++++	++++	++++	++++
Concolor	+++	++++	++++	++++	++++
Mojave	+++	++++	++++	++++	++++
C. d. terrificus venom	+++	++++	++++	ND	ND
C. s. scutulatus venom	+++	++++	++++	ND	ND
PL A2 *C. atrox*	+	+	++	ND	ND
PL A2 *C. adamantous*	+	+	++	ND	ND
β-Bungarotoxin	trace	trace	trace	ND	ND
Neurophysin	-	-	-	ND	ND
PL A2 Honey Bee	-	-	-	ND	ND
PL A2 *Naja naja atra*	-	-	-	ND	ND

<1/500 -
1/500 trace
1/12,500 +
1/64,000 ++
1/256,000 +++
1/1,000,000 ++++
ND = Not Determined

Reactions were scored by using the greatest dilution that provided an above background reading in a serial dilution experiment.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBO
	DA 1522	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. CHANGE	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101	3M161102BS12	AB	002
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Aerosol Exposure Technology

12. SUBJECT AREAS 1503 Defense; 0605 Clinical Medicine; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	2.0	453
c. TYPE	d. AMOUNT	86		
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Airborne Diseases Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Stephenson, E H
d. TELEPHONE NUMBER (include area code) 301-663-7453	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Young, H W
MILITARY/CIVILIAN APPLICATION M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) York, C G

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Aerosols; (U) Military Medicine; (U) BW Defense; (U) Bacteria

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Studies of airborne-induced infections and intoxications necessitate a variety of aerosol exposure systems be available to accommodate varying research requirements. Equipment is identified or developed to meet the unique requirements to define the pathogenesis and immunogenesis of airborne infections or intoxications, and to evaluate therapeutic and prophylactic regimens developed to protect US military personnel.

24. (U) Overt efforts are made to identify commercially available equipment that will enhance and expand the aerosol exposure capabilities. When commercial equipment is not available, new systems are designed and developed in-house.

25. (U) 8410-8509 - An improved nose-only exposure system was designed, the prototype tested, and the final system is being manufactured. Results of the characterization showed that there are no significant differences in aerosol concentration or particle size distribution among the 28 nose ports. A Hazelton H-1000 cabinet was manufactured to incorporate specific modifications. The cabinet proved useful and successful as a mobile P-2/3 level containment facility that can be used in all levels of containment laboratories. A head-only monkey exposure chamber that will accommodate 1- to 10- kg monkeys was designed and tested.

BODY OF REPORT

PROJECT NO. 3M161102BS12: Science Base/Medical Defense Against BW

WORK UNIT NO. S12-AB-002: Aerosol Exposure Technology

PRINCIPAL INVESTIGATOR: E. H. Stephenson, D.V.M., Ph.D., COL, VC

ASSOCIATE INVESTIGATORS: H. W. Young
C. G. York

Background:

Studies of airborne-induced infections and intoxications necessitate that a variety of aerosol exposure systems be available to accommodate the varying research requirements. In the past decade, exposures of rodents or small, non-human primates (< 1 kg) at the Institute were accomplished by using a whole body exposure system (1) with either a Collison or Devilbiss nebulizer. Overt efforts are ongoing to identify commercially available equipment that will enhance and expand the aerosol exposure capabilities. When commercial equipment is not available, new systems are designed and developed in-house.

Summary:

An improved nose-only exposure system was designed, the prototype tested. The final system is being manufactured. Results of the characterization showed that there were no significant differences in aerosol concentration or particle size distribution among the 28 nose ports. A Hazelton H-1000 cabinet was manufactured to incorporate specific modifications. The cabinet proved to be useful and successful as a mobile P-2/3-level containment facility. Further modifications to the cabinet are being made, whereby it can be used for animal exposures within a P-4 suite. A head-only, monkey exposure chamber that will accommodate 1- to 10-kg monkeys was designed and tested. The compilation of a data base on the performance of available aerosol dissemination and sampling devices is ongoing; this will provide useful information on our immediate capabilities for aerosol exposure systems.

Progress:

An improved nose-only exposure system was designed and fabricated. The prototype system was received and evaluated for performance with a small particle aerosol by using two different dissemination devices, a 3-jet Collision and a modified 1-jet Collision. The 3-jet Collision nebulizer was used in the characterization of 17.5- and 22.5- L/min systems and the modified 1-jet Collison nebulizer was used in the characterization of a 7.0- L/min system.

The design criterion for the improved nose-only exposure system included (a) the presentation of the same concentration of fresh aerosol to each animal, (b) the minimization of the effects of leakage around the animal's nose, (c) the accommodation of a range of sizes of several species of animals, and (d) the incorporation of an animal restraint system that minimizes stress during exposure. To achieve these criteria, each port was maintained at atmospheric pressure to reduce aerosol leakage around the nose seal, and three different sizes of animal restraint devices were designed in order to accommodate mice, rats, and guinea pigs.

The aerosol was sampled at the inlet and at each of the 28 exposure ports in three replicate tests by all-glass impingers and a cascade impactor. We found that there were no significant differences in either aerosol concentration or particle size distribution among exposure ports.

A Hazleton H-1000 cabinet, modified to our expectations, was received. The modified Hazleton cabinet met its proposed application as a mobile, P-2/3 level containment facililty which can be transported, with the appropriate aerosol exposure system inside, to various locations. The modified Hazleton cabinet was utilized successfully in a collaborative study with the NIH to expose squirrel monkeys to a virus. The aerosol system was a two-chamber configuration accommodating four squirrel monkeys per trial. The modified Hazleton cabinet is currently being modified further for a collaborative study with Ebola virus in a P-4 containment suite.

A head-only, monkey exposure apparatus was designed and characterized. Although the apparatus accomodates only one monkey per trial, it will hold a range of sizes of monkey. It allows the monkey to lie on its back with only its head in the exposure chamber.

An extensive data base was compiled on the performance of various aerosol dissemination and sampling devices. Aerosol dissemination devices tested include the Vaponefrin nebulizer, a modified 1-jet Collision nebulizer, a number of Devilbiss 40 nebulizers, and a vibrating orifice aerosol generator. The sampling devices tested include a 3.0- L/min glass impinger; 2.0-, 1.0-, and 0.1- L/min Mercer 7 Stage Cascade Impactors; several types of filters; and a Royco 4102 Particle Sizer. This data base will provide the information needed for assembly of various aerosol exposure systems. This compilation of data is ongoing as new and improved devices are found.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBO
				DAOG 1526	85 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS12	AC	003
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE *(Precede with Security Classification Code)* (U) Biology of Viral Agents of Potential BW Importance

12. SUBJECT AREAS 1503 Defense; 0605 Clinical Medicine; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		85	5.3	1519
c. TYPE	d. AMOUNT	86	0.0	0*
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Dalrymple, J M
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7241
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Smith, J F
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Viral Diseases;(U) Prophylaxis;(U) Lab Animals;(U) Vaccines;(U) Mice;(U) Guinea Pig;(U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) To elucidate antigenic composition and replicative mechanisms of selected toga-, bunya-, and arenaviruses unique in their capacity to cause widespread epidemics of human disease. To characterize viral constituents to identify immunogens of potential prophylactic value. Define virus-specific replication mechanisms as targets for antiviral therapy. To provide a technical base on which to formulate a better BW defense program.

24. (U) Characterize the structural protein antigens of selected pathogens by biochemical and biophysical separation methods and define specific antigenic determinants of these proteins using specific antibodies. Characterize nucleic acids of selected virus pathogens to aid in their type-specific identification, deduce their replicative strategy, and ultimately evaluate various methods for disease control.

25. (U) The antigenic composition of Rift Valley fever virus (RVFV) has been defined by purifying each of the three major structural proteins, and utilizing these proteins as immunogens and antigens to produce and characterize antigen-specific polyclonal sera and monoclonal antibodies. Six distinct vaccine-relevant antigenic determinants (epitopes) capable of eliciting in vitro neutralizing antibodies have been identified on the G1 and G2 surface viral glycoproteins by competitive binding assays. Passive immunization of mice with monoclonal or polyclonal antibodies, however, have shown that only G2-specific neutralizing antibodies provide significant protection in vivo. Three distinct in vivo protecting determinants have each been mapped to specific amino acid sequences on the G2 polypeptide. Immunization of mice with G2 gene products expressed in *E. coli* as well as recombinant vaccinia viruses containing RVFV glycoprotein genes have been shown to protect mice from a lethal challenge of RVFV.

*This research will be part of S12AB in FY 86.

BODY OF REPORT

PROJECT NO. 3M161102BS12: Science Base/Medical Defense Against BW

WORK UNIT NO. S12-AC-003: Biology of Viral Agents of Potential BW Importance

PRINCIPAL INVESTIGATOR: J. M. Dalrymple, Ph.D.

ASSOCIATE INVESTIGATOR: J. F. Smith, Ph.D.

Background

The major research efforts pursued under this work unit have been: definition of the antigenic determinants of Rift Valley fever virus (RVFV) that induce protective immunity, and testing experimental antigens to assess their relative efficacy in providing protection to animals. These studies have been carried out concurrently with collaborative efforts via extramural contracts. These collaborative studies have been successful in cloning and sequencing the Rift Valley fever virus genes that code for the two virion surface glycoproteins, G1 and G2. These viral antigens are now known to carry several, if not all, of the protective determinants. Identification of these protective epitopes by cloning genes, determining nucleotide sequences, and monoclonal antibody analysis constitute the necessary data base required to design and construct immunogens. The overall objective is to produce a vaccine with significant advantages over the existing, inactivated, whole-virus vaccine. A secondary objective is to establish the techniques and capabilities required for these constructions so that these procedures can be extrapolated for use with other viral agents for which no immunoprophylaxis exists.

Summary:

The antigenic composition of Rift Valley fever virus has been defined by purifying each of the three major structural proteins and utilizing these proteins as immunogens and antigens to produce and characterize antigen-specific, polyclonal sera and monoclonal antibodies. Six distinct vaccine-relevant, antigenic determinants (epitopes), capable of eliciting in vitro neutralizing antibodies, have been identified on the G1 and G2 surface viral glycoproteins by competitive binding assays. However, passive immunization of mice with monoclonal or polyclonal antibodies has shown that only G2-specific, neutralizing antibodies provide significant protection in vivo. Three distinct protective (in vivo) determinants have been mapped to specific amino acid sequences on the G2 polypepetide. Immunization of mice with G2 gene products expressed in *E. coli*, as well as recombinant vaccinia viruses containing Rift Valley fever virus glycoprotein genes, has been shown to protect mice from a lethal Rift Valley fever challenge.

Progress:

Purification and Antigenic Characterization of Rift Valley Fever Structural Proteins (G1, G2, NC) For the production of monospecific, polyclonal antisera to each of the three major structural proteins, gradient-purified virus was solubilized initially in nondenaturing detergents and the resulting lysate fractionated by equilibrium density gradient centrifugation in cesium chloride. In these gradients, the nucleocapsid complexes banded near the bottom of the gradients (ρ=1.31), leaving the two glycoproteins unresolved in the supernatant fractions. The nucleocapsid and glycoprotein fractions were not detectably cross-contaminated or contaminated with host cell proteins. The two glycoproteins were separated by preparative (column) isoelectric focusing. This procedure is highly efficient as the two glycoproteins have widely divergent isoelectric points (Pi=4.5 versus 9.5). The purified nucleocapsid complexes, as well as the glycoprotein fractions, were emulsified individually in complete Freund's adjuvant, and inoculated i.p. and s.c. into adult BALB/c mice. The mice were then boosted with soluble antigens at 30 days, and ascites were induced by the inoculation of sarcoma 180 cells i.p.

The specificity of the ascitic fluids was determined by immunoprecipitation and polyacrylamide gel electrophoresis as well as by ELISA assays with the purified viral proteins as antigens. Each of the ascitic fluids was found to be absolutely specific for the immunizing protein, confirming the purity of the immunogens in the initial biochemical separations. As indicated in Table 1, these ascitic fluids were also characterized by indirect immunofluorescence (IFA), hemagglutination inhibition (HI), and plaque-reduction neutralization (PRN). Results demonstrated that these ascitic fluids have essentially equivalent titers in ELISA and IFA assays and show no reactivity with uninfected VERO cells by IFA. Hemagglutination inhibition assays demonstrated that high-titer activity was present in both anti-G1 and anti-G2 preparations but absent in anti-NC sera. From the standpoint of producing protective immunogens, the most interesting observation is that both G1 and G2 polypeptides clearly contain virus-neutralizing determinants with significantly higher titers associated with anti-G1 ascitic fluids.

These antisera were tested for their relative efficacy in providing protection in vivo in a mouse model (Table 2). In these studies, control and antigen-specific ascitic fluids were used to immunize passively adult C57 mice 24 h prior to challenge. As expected, standard hyperimmune ascitic fluid, which contained a high neutralization titer as well as reactivity to all RVFV proteins, protected all test animals; whereas negative control ascitic fluid or anti-NC ascitic fluid provided no protection. However, the observation that anti-G1 preparations, despite their high in vitro neutralization titers, failed to protect, whereas anti-G2 antibodies consistently protected all challenged animals, was unexpected. Although several possibilities exist, the reason(s) why neutralizing anti-G1 antibodies failed to protect in vivo currently remain untested and unclear. However, as shown below, similar conclusions have been obtained from analysis of RVFV-specific, monoclonal antibodies.

Production and Characterization of Monoclonal Antibodies to RVFV G1 and G2 Initial attempts to produce monoclonal antibodies to phleboviruses resulted in the isolation of hundreds of hybridomas secreting antibodies to the internal nucleocapsid protein, which is apparently the immunodominant viral antigen. Although such antibodies are useful for diagnostic procedures as well as virus-strain comparisons (5), they are not useful for the identification of protective epitopes which are expressed on the surface glycoproteins, G1 and G2. Consequently, a total of five fusions have been carried out in which BALB/c mice were immunized and boosted with glycoprotein-enriched fractions obtained from purified virus or with purified G1 or G2. A total of 50 glycoprotein-specific monoclonal antibodies have been characterized to date. This characterization has included: 1) antigen specificity; 2) isotype determination; 3) IFA, HI, and ELISA titration; 4) competitive binding analysis; 5) in vitro neutralization capacity; and 6) in vivo protective capacity, as monitored by passive immunization and subsequent challenge of C57BL/6 mice. The results obtained with selected monoclonal antibodies are shown in Tables 3 and 4.

These data show that G2-specific antibodies which demonstrate in vitro neutralization capacity are capable of offering absolute protection in passive immunization experiments. Non-neutralizing, G2-specific monoclones (from five distinct competition groups) failed to provide significant protection. None of the G1-specific monoclonal antibodies (encompassing seven competition groups) efficiently protected passively immunized animals, despite the fact that milligram amounts of antibody were transferred, and that several of these antibodies showed in vitro neutralization titers of 1600 to 25,600. There remains the possibility (if not probability) that additional protective determinants exist for which no corresponding monoclone has yet been isolated. However, these results are in total agreement with those obtained with the antigen-specific, polyclonal sera, which likely contained antibodies reactive with most, if not all, of the major epitopes expressed on these proteins.

Mapping of Protective Epitopes and Selection of Peptides to be Synthesized

Mapping Various approaches of mapping epitopes (defined by monoclonal antibodies) to specific amino acid sequences have been attempted in various virus sytems including: 1) monitoring reactivity with protease-derived fragments; 2) comparing nucleotide sequences of parental and antibody-induced virus variants; and 3) monitoring antibody reactivity with randomly produced synthetic peptides. Although apparently successful in some cases, these approaches have been unsuccessful or even misleading in many instances. The procedures adopted by Keegan and Collett (6) (via extramural contract with Molecular Genetics) have utilized a different approach in which "nested sets" of defined polypeptides have been expressed in *E. coli* coded by RVFV-specific genetic inserts of different lengths. Plus/minus reactivity of a monoclonal antibody with these known polypeptides defined the epitope to a specific sequence provided that allosteric effects were absent. The epitope is assumed to lie within the end sequences of the longer of two polypeptides showing plus/minus reactivity. Utilizing these techniques, six protecting, G2-specific monoclonal antibodies (characterized and supplied by USAMRIID) have been mapped to three sequences on G2 of 11, 19, and 32 amino acid residues. The total number of amino acid residues in G2 is not precisely known, but is approximately 500. All three of these epitopes lie within 135 amino acid residues in the N terminal half of G2. The available data, therefore, suggest that vaccine-relevant epitopes are not only restricted to G2, but are confined to a 135-residue sequence - a length well within the capabilities of current technology to examine adequately by synthetic peptide analysis.

RVFV, therefore, constitutes perhaps the best virus model defined to date to test the feasibility of the synthetic peptide approach to vaccine development. Not only are several protecting epitopes precisely mapped but several of these epitopes are known to be conformation-independent.

Selection of Peptide Sequences X-ray crystalographic studies of myeloma proteins have shown that antibody-combining sites are approximately 18 angstroms - indicating that a protein epitope can not exceed 5 to 6 amino acid residues. In practice, synthetic peptides are usually synthesized from 9 to 16 residues. The potential number of such peptides from the 135-residue sequence described above (assuming any start position from 1 to 126) is finite but unmanageably large. There are currently two procedures which are used to select specific sequences most likely to result in an immunogenic peptide inducing antibodies reactive with a native protein.

The first identifies hydrophilic and hydrophobic domains along the linear sequence and assumes that hydrophilic sequences will be at the surface of a globular protein and, therefore, potential antibody-combining sites. The second procedure utilizes the Chou and Fasmen algorithm to predict secondary structure and focuses on "turn" sequences between "alpha helical" and "beta sheet" structures. This procedure relies on observations from x-ray crystallography that such turns are invariably at the surface of globular proteius and are often immunodominant epitopes.

Although these computer-assisted analyses may not be completely accurate, the sequences of the I-4D4 epitope, which has been most precisely mapped, could have been predicted to be a potentially ideal epitope, based on these criteria. There are, in addition, several other hydrophilic domains which may represent important epitopes for which no corresponding monoclone was isolated. Based on these data, 11 synthetic peptides have been selected and are currently being synthesized at Meloy Laboratories via an extramural contract. Nine are within the 135-residue sequence and two are located within sequences preceding G2. These peptides will be convalently coupled to keyhole limpet hemocyanin and bovine serum albumin and used to immunize rabbits and mice. Resulting antisera will be assessed for their relative ability to bind and/or neutralize RVFV. In addition, mice immunized with synthetic peptides will be subsequently challenged with RVFV to determine the level of protection induced.

PRESENTATIONS:

1. **Smith, J. F., R. Lanciotti, and D. Pifat.** Analysis of the intracellular synthesis and antigenic characteristics of Rift Valley fever virus glycoproteins. Presented at the International Meeting on Negative Stranded Viruses. Cambridge, England, 15-20 September 1985.

2. **Alonzo-Caplen, F. V., and J. M. Dalrymple.** Glycosylation and transport of RVFV envelope glycoproteins. Presented at the International Meeting on Negative Stranded Viruses. Cambridge, England, 15-20 September 1985.

3. **Collette, M. S., K. Keegan, S-L. Hu, P. Sridhar, A. F. Purchio, W. H. Ennis, and J. M. Dalrymple.** Protective subunit immunogens of Rift Valley fever virus from bacteria and recombinant vaccinia virus. Presented at the International Meeting on Negative Stranded Viruses. Cambridge, England, 15-20 September 1985.

4. **Smith, J. F., D. Pifat, and R. Lanciotti.** Synthesis and antigenic structure of Rift Valley fever virus glycoproteins. Presented at the Sixth International Congress of Virology. Sendai, Japan, 1-7 September 1984.

5. **Pifat, D., and J. F. Smith.** Characterization of monoclonal antibodies to Punta Toro virus: requirements for passive protection in-vivo. Presented at the Sixth International Congress of Virology. Sendai, Japan, 1-7 September 1984.

6. **Knauert, F. K., M. D. Parker, and J. M. Dalrymple.** Use of cDNA probes to detect Rift Valley fever virus. Presented at the American Society for Virology Annual Meeting. Albuquerque, New Mexico, 21-25 July, 1985.

7. **Smith, J. F., R. S. Lanciotti, and W. H. Ennis.** Antigenic analysis of protective epitopes on Rift Valley fever virus surface glycoproteins. Presented at the American Society for Virology Annual Meeting. Albuquerque, New Mexico, 21-25 July 1985.

8. **Pifat, D. Y., and J. F. Smith.** Characterization of protective monoclonal antibodies specific for Punta Toro virus. Presented at the American Socity for Virology Annual Meeting. Albuquerque, New Mexico, 21-25 July 1985.

PUBLICATIONS:

1. **Collette, M. S., A. F. Purchio, K. Keegan, S. Frazier, W. Hays, D. Anderson, M. Parker, C. Schmaljohn, J. Schmidt, and J. M. Dalrymple.** 1985. Complete nucleotide sequence of the M RNA segment of Rift Valley fever virus. *Virology* **144**:228-245.

2. **Ihara, T., J. F. Smith, J. M. Dalrymple, and D. H. L. Bishop.** 1985. Complete sequences of the glycoproteins and M RNA of Punta Toro phlebovirus compared to those of Rift Valley fever virus. *Virology* **144**:246-259.

3. **Tappert, H. I., J. M. Meegan, J. F. Smith, J. M. Dalrymple, and C. J. Peters.** Monoclonal antibodies to Rift Valley fever virus: characterization and detection of antigenic variation in geographically different virus strains. Submitted to *J. Gen. Virol.*

LITERATURE CITED

1. **Tappert, H. I., J. M. Meegan, J. F. Smith, J. M. Dalrymple, and C. J. Peters.** Monoclonal antibodies to Rift Valley fever virus: characterization and detection of antigenic variation in geographically different virus strains. Submitted to *J. Gen. Virol.*

2. **Keegan, K., and M. S. Collette.** 1986. Mapping of antigenic determinants on the G2 glycoprotein of Rift Valley fever virus. Submitted to *J. Virol.*

Table 1 Characteristics[a] of Anti-RVFV G-1, G-2, and NC polyclonal ascitic fluids

	ELISA				IFA-VERO				
IMMUNOGEN	VIRION	NC	G-1	G-2	INF	UNINF	HI	PRNT-50	PRNT-80
NC	12,800	12,800	<50	<50	4200	<8	20	<10	<10
G-1	15,500	<50	21,900	<50	1660	<8	3200	10,240	2560
G-2	11,800	<50	<50	12,800	1530	<8	3280	2,560	80

[a]Values represent arithmatic mean titers from 8 independently derived ascitic fluids.

Table 2 Passive immunization[a] with RVFV antigen-specific polyclonal antisera

ANTIBODY	ANTIGEN SPECIFICITY	PRNT 50%	PRNT 80%	ELISA	PROTECTION
None (S-180)	-	<10	<10	<50	0/10 (0%)
Hyperimmune	All	10,240	10,240	50,000	20/20 (100%)
PCG1	G1	10,240	2,560	12,800	1/20 (5%)
PCG2	G2	2,560	80	12,800	18/20 (90%)
PCNC	NC	<10	<10	50,000	0/20 (0%)

[a]Mice were passively immunized with 0.2 ml of undiluted ascitic fluid 24 h before challenge.

Table 3 Passive Immunization[a] of C57BL/6 Mice with G2-specific monoclonal antibodies

MCAb	PRNT 80%	COMP. GROUP	ISOTYPE	ELISA	PROTECTION
None (Sp2/0)	<10	-	-	<50	0/20 (0%)
R1-4D4	10,240	F	G_1,k	250,000	10/10 (100%)
R10-10A	1,280	F	G_{2a},k	100,000	10/10 (100%)
R5-3G2	160	F	G_1,k	100,000	25/25 (100%)
R5-9B6	40	ND	M,k	10,000	19/20 (95%)
R1-3C10	<10	D	G_1,k	250,000	3/20 (15%)
R1-3D10	<10	C	G_1,k	250,000	0/10 (0%)
R2-3D11	<10	E	G_{2b},k	100,000	3/25 (12%)
R2-1E5	<10	ND	G_1,k	100,000	0/10 (0%)
R5-3C11	<10	E	G_1,k	100,000	4/27 (14%)
R2-1E4	<10	ND	G_1,k	250,000	4/25 (16%)
R1-1F11	<10	ND	G_1,k	250,000	0/10 (0%)
R4-3D11	<10	ND	G_1,k	100,000	0/17 (0%)

[a]Mice were passively immunized with 0.2 ml of undiluted ascitic fluid 24 h before challenge.

Table 4 Passive immunization[a] of C57BL/6 mice with G1-specific monoclonal antibodies

MCAb	PRNT 80%	COMP. GROUP	ISOTYPE	ELISA	PROTECTION
None (SP2/0)	<10	-	-	<50	0/20 (0%)
R1-4B6	25,600	B	G_1,k	250,000	4/27 (15%)
R4-5E2	25,600	ND	G_1,k	250,000	1/20 (5%)
R1-5G2	25,600	D	G_{2a},k	500,000	6/17 (35%)
R1-3B4	1,600	F	G_1,k	250,000	5/27 (18%)
R4-4G11	1,600	ND	G_1,k	250,000	5/27 (18%)
R1-1G6	1,600	A	G_1,k	250,000	0/10 (0%)
R4-6G4	1,600	ND	G_1,k	75,000	4/27 (15%)
R2-1E5	<10	E	G_1,k	500,000	0/10 (0%)
R2-1F7	<10	C	G_1,k	500,000	0/10 (0%)
R2-1G4	<10	E	G_1,k	500,000	0/10 (0%)
R1-P5E8	<10	G	G_{2b},k	100,000	0/10 (0%)
R4-3B9	<10	ND	G_1,k	100,000	0/10 (0%)
R4-7D2	<10	ND	G_1,k	100,000	0/10 (0%)

[a]Mice were passively immunized with 0.2 ml of undiluted ascitic fluid 24 h before challenge.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DAOG-1529	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SY DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY H. TERM	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS12	AD	004
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Pathophysiology of Toxemias/Diseases of Potential BW Importance

12. SUBJECT AREAS 0613 Microbiology; 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10		DA	C. In-House

17. CONTRACT/GRANT
- a. DATE EFFECTIVE — EXPIRATION
- b. CONTRACT/GRANT NUMBER
- c. TYPE — d. AMOUNT
- e. KIND OF AWARD — f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thous
85	2.8	778
86	0.0	0*

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathophysiology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Wannemacher, R W, Jr.
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7181
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Bunner, D L
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) BW Defense; (U) Low Molecular Weight Toxins; (U) Metabolism; (U) Infectious Disease; (U) Rapid Detection; (U) T-2 Toxin; (U) RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop basic program on detection and mechanism of action of low molecular weight toxins and evaluate mechanism of enhancement of defense for US military personnel who may be exposed to toxins of biological origin.

24. (U) Develop animal models for basic studies on pathological, biochemical, immunological, and physiological alterations produced by toxins. Develop procedures for extraction of toxins from environmental or physiological samples and detect them by biological, chemical, or immunological assays.

25. (U) 8410-8509-To provide medical defense against these toxins, studies have been involved in elucidating detection, pathophysiology, toxicology, prophylaxis, and treatment of intoxications. An HPLC-mass spectrometry procedure has been developed for detection of saxitoxin in biological fluids. Differences were noted in metabolism and pharmacokinetics of T-2 mycotoxins when adminstered parenterally or dermally. While T-2 tetraol was the major metabolite in biological fluids, the parent T-2 could be detected only after dermal exposure. Data from in vitro studies on inhibition of protein synthesis did not correlate with data from mouse lethality studies, which raises some doubt whether protein synthesis inhibition is the major mechanism of action. This conclusion is supported by the inability to detect this inhibition after a lethal dose of T-2 was administered to a rat.

*This research will be part of Work Unit S12AA in FY 86.

BODY OF REPORT

PROJECT NO. 3M161102BS12: Science Base/Medical Defense Against BW

WORK UNIT NO. S12-AD-004: Pathophysiology of Toxemias/Diseases of Potential BW Importance

PRINCIPAL INVESTIGATOR: Robert W. Wannemacher, Jr., Ph.D.

ASSOCIATE INVESTIGATORS: David L. Bunner, COL, M.D., MC
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Background:

A number of low molecular weight toxins of biological origin have been implicated as potential biological warfare agents. These toxins include certain mycotoxins (trichothecenes), sodium channel blocker agents (saxitoxin, tetrodotoxins), other toxins of marine origins, blue-green algae toxins, and other low molecular weight toxins of plant and animal origin. The trichothecene mycotoxins have been detected in the environment and physiological fluids of individuals exposed to "yellow rain" (1). Most of the published literature on these toxins involves studies on orally ingested contaminated grains and seafood (2,3). In ord to develop a medical defense against these toxins, a basic technology program was initiated on the rapid detection, pathophysiology, and cell mechanism of action.

Summary:

To provide medical defense against these toxins, studies have been involved i elucidating detection, pathophysiology, toxicology, prophylaxis, and treatment of intoxications. An HPLC/mass spectrometry procedure has been developed for detectic of saxitoxin in biological fluds. Differences were noted in metabolism and pharmacokinetics of T-2 mycotoxins when administered parenterally or dermally. While T-2 tetraol was the major metabolite in biological fluids, the parent T-2 was detected in these fluids only after dermal exposure. Data from in vitro studies on the inhibiton of protein synthesis did not correlate with data from mouse lethality studies, which raises some doubt whether protein inhibition is the major mechanism in T-2 mycotoxicosis. This conclusion is supported by our inability to detect this inhibition when a lethal T-2 dose was given to a rat.

Progress:

Detection A summer faculty member (Dr. Hines) has developed techniques capable of detecting STX in complex mixtures (e.g., urine) on the basis of structure. An HPLC technique, similar to that of Sullivan et al. (4), was used to purify saxitoxin from guinea pig urine. STX in the presence of an ion-pairing reagent (heptanesulfonic acid) was retained by a C-18 reverse-phase column and was eluted a a well-defined and symmetrical peak by a solvent system containing 20% acetonitrile. This peak was identified as STX by a fluorescence method (5). Also, mass spectral analysis of the HPLC peak positively identified it as STX. The mass spectrometry procedure was Fast Atom Bombardment (FAB), with selective ion monitoring, which could detect 10 ng STX. This technique should allow future studies on the metabolism and pharmacokinetics of STX. This method will be important for detection and confirmation of STX present in biological samples from suspected biological warfare attack.

Pharmacokinetics and Metabolism In a previous study, [^{3}H]T-2 was administered i.m. to guinea pigs, and the fate and distribution of T-2 and metabolites was followed over 28 days. Recently, we have identified the metabolites appearing in plasma, bile, urine, and five major tissues. By 30 min, tissues showed 25% of the radioactivity; and extractable metabolites were identified as T-2, 3'OH-T-2, HT-2, 3'OH-HT-2, and T-2 tetraol. Within 5 days, 75% of the total radioactivity was excreted in urine and feces at a ratio of 4:1. Urinary metabolites were quantitatively identified as T-2 tetraol, 4-deacetyl-neosolaniol (DANS), 3'OH-HT-2, and several unknowns. Biliary metabolites were identified as HT-2, DANS, 3'OH-HT-2, 3'OH-T-2 triol, and several unknowns. Hydrolysis of the polar unknowns with β-glucuronidase yielded 80% HT-2, 11% 3'OH-HT-2, and 1% T-2 tetraol. By 30 min, 2% of the total radioactivity was found in plasma. Metabolites in plasma over the first 24 h were estimated to be HT-2 (5-40%), DANS (7-10%), T-2 tetraol (3-19%), and polar conjugates (12-40%). The parent T-2 toxin was not detected in plasma, urine, or bile (detection limit, 1 μg/nml). Results indicate that radioactivity was still present in tissues and body fluids 28 days after exposure to the toxin, and that urine, rather than blood, and T-2 tetraol, rather than T-2, were the sample and metabolite of choice for diagnostic testing.

The fate and distribution of [^{3}H]T-2 toxin were assessed in guinea pigs and rats that were dosed topically with T-2 in either DMSO or methanol. In the guinea pig study, when [^{3}H]T-2 was applied in methanol, 80% remained in or on the skin after 28 days, but when applied in DMSO, less than 5% remained. This suggests that in the methanol group the dermal layer may be acting as a reservoir for the toxin. More toxin was absorbed at all time points in the DMSO group; however, the rates of absorption, expressed in terms of halftimes to maximum absorption, were comparable. Approximately 50% of the radioactivity appeared in urine of the DMSO group over 28 days, while only 5% was in the urine of the methanol group. Radioactivity peaked in blood and bile by day 1 in the DMSO group and by day 3 in the methanol group, suggesting the rate of uptake into these compartments was faster when DMSO was the delivery solvent. While a greater amount of toxin was absorbed in the DMSO group, a larger percent of that absorbed was found in tissues in the methanol group. This, coupled with the fact that, in the methanol group, only 5% of the absorbed dose was excreted over 28 days, suggests that the rate of elimination was slower in the methanol group. In the methanol group, T-2 was still detectable in urine and bile 6 days post-exposure and was conjugated as glucuronides. T-2 was present in the plasma of the methanol group at day 3 and in the DMSO group through day 28. In the DMSO group, less than 1% of the urinary and biliary radioactivity was associated with the parent T-2 or conjugated T-2. This suggests that T-2 was metabolized more efficiently when delivered in DMSO as compared to methanol.

An in vitro model was used to study the rate of penetration through and metabolism of [^{3}H]T-2 by excised human, monkey, and guinea pig skin. Total percent penetration was 0.33% and 0.13% for whole human and monkey skin, respectively. Radioactive compounds in the receptor fluid bathing human skin corresponded to T-2 (69%) and HT-2 (25%). Although penetration was faster through monkey skin than through human skin, freezing the skin sample increased the penetration in both cases. The dose-response experiments indicate that exposure to higher doses of T-2 results in an increased efficiency of penetration.

<u>Cell Biology</u> Nineteen 12,13-epoxytrichothecene mycotoxins were tested for their relative protein synthesis inhibition effects in Vero cells and rat spleen lymphocytes. Although the lymphocytes were generally more sensitive to the mycotoxins, good correlation existed between the relative toxicities of the various trichothecenes in the two cell systems. The most cytotoxic mycotoxins (T-2, verrucarin A, and roridin A) have acetyl side groups on or a hydrocarbon chain between carbon 15 and carbon 4 of the basic ring structure. Loss of side groups from either of these positions or an isovaleryl group at carbon 6, resulted in reduced cytotoxicity [T-2 to HT-2, neosolaniol or diacetoxyscirpenol (DAS)]. Any combination of loss from all three positions further weakened the cytotoxic effect of the mycotoxins [T-2 triol, T-2 tetraol, 15-monoacetyl DAS (MAS), scirpenol, fusarenon X, and deoxynivalenol (DON)]. Reduction of the hydroxyl groups of these compounds to hydroxides (verrucarol and deoxyverrucarol) lowered the cytotoxic level to over three logs less than the most cytotoxic mycotoxins. Addition of side groups resulting in reduced cytotoxicity occurred only when an acetyl group was added to the C3 position of T-2 (acetyl T-2) and DON (3-acetyl DON), or when an epoxide was substituted across the 9,10 carbons of DAS (β-epoxide DAS). Mixtures of these and other mycotoxins indicated that their cytotoxic effects were additive and showed no synergism or competition for binding to the active site. When this in vitro cytotoxicity was compared on a structure-function relationship to mouse lethality, discrepancies were observed. In the mouse bioassay, only a one-log difference was observed in the toxicity of various trichothecenes that were tested. In contrast, a 2- to 3-log difference was observed in cytotoxicity when the same trichothecenes were evaluated in the in vitro cell system. Several of the trichothecenes, such as acetyl T-2, T-2 tetraol, and nivalenol, were very weak inhibitors of protein synthesis in cell systems but had an in vivo toxicity similar to that of T-2 toxin. Furthermore, the administration route of certain mycotoxins (T-2 and verrucarin A) could markedly influence the toxicity in the mouse lethality assay. Thus, the in vitro cell system is not an accurate evaluation of the toxicity of a given trichothecene in whole animal studies.

The recent availability of labeled mycotoxins, other than T-2, has resulted in several studies of interest. The rate of uptake of an equal concentration of T-2, HT-2, tetraol, and triol into Vero cells and lymphocytes was directly proportional to their cytotoxic level. Competition studies of cold mycotoxins in the presence of [^{3}H]T-2 also showed the rate of uptake of [^{3}H]T-2 was only reduced in the presence of enough of the cold mycotoxin that would normally be cytotoxic. This may be in great excess of the T-2 concentration for less potent mycotoxins. This was another indication of mycotoxin transport being an important factor in cell-level cytotoxicity.

Further studies on the reversibility/recovery effects of the macrocyclics as opposed to the other mycotoxins have been carried out with the recent availability of the labeled macrocyclic, verrucarin A. Release of verrucarin A from Vero cells after loading for 1 h was compared to release of T-2 mycotoxin. Over a 2-h period, more than 50% of the T-2 was released by the cells, while less than 20% loss of the verrucarin A occurred in the same time period. Additional studies on uptake rates and metabolism in various cell systems will help to determine the basic differences between the macrocyclics and other trichothecene mycotoxins.

A wide variety of culture cell lines, in addition to primary cells like lymphocytes, macrophages, hepatocytes, intestinal cells, and heart cells, have been utilized in protein synthesis inhibition assays. More recently, the L6 cells T-2 and verrucarin A sensitivity was assessed. T-2 had an ED_{50} of .0034 μg/ml and verrucarin A .0028 μg/ml, with the minimal effective dose of less than .001 μg for either T-2 or verrucarin. This is 3 to 4 times more sensitive than other cell systems so far studied.

Studies on metabolism of [^{3}H]T-2 have been completed in hepatocytes, rat intestinal cells, lymphocytes, heart cells, and Vero cells, with the highest to lowest rates of metabolism in the order of cell types given. Because the hepatocytes demonstrated the greatest rate of metabolism, additional studies are in progress employing media from T-2 exposed cultures of hepatocytes and various subfractions of the cells. Both the media and, to varying degrees, the various subfractions of hepatocytes have the ability to metabolize T-2 to HT-2. However, only intact cells form other intermediates and, eventually, all polar metabolites that remain at the origin on the TLC plates. Initial results from metabolism of [^{3}H] verrucarin A in hepatocytes and heart cells indicate that it is metabolized just as well, if not more rapidly, than T-2. This is in opposition to the hypothesis that the more toxic nature of the macrocyclics is due to the inability of cells to metabolize and release these compounds.

Studies were performed to determine the stability of T-2, HT-2, and T-2 tetraol in urine and blood at various storage temperatures, and with or without sodium fluoride over six months. To date (one month), the following conclusions can be drawn: (1) As was expected, all toxins are more stable when stored at -70 C than at room temperature, but T-2 toxin did degrade. (2) Tetraol was stable in both urine and blood, regardless of storage temperature or the addition of sodium fluoride. (3) HT-2 was the least stable metabolite. (4) The addition of sodium fluoride only slightly increased the stability of T-2 in blood, but had no effect on the stability of the other toxins in blood or urine.

T-2 toxin is a known inhibitor of eukaryotic protein synthesis. T-2 toxin has been shown to inhibit mitochondrial protein synthesis, a prokaryotic-like system. An in vitro system employing cycloheximide, an inhibitor of cytoplasmic protein synthesis, and stimulated by a S-100 supernatant from rat liver, was used to characterize the mitochondrial protein synthesis system. Energy requirements were satisfied by the addition of an external ATP-generating system. Amino acid incorporation into protein was dependent on the concentration of mitochondria and was inhibited by chloramphenicol. The rate of uptake of [^{3}H]leucine into mitochondria was unaffected by the addition of T-2 and was not the rate-limiting step in incorporation. The rate of protein synthesis was decreased 70% by 0.03 μg/ml T-2 toxin. Other trichothecene mycotoxins (HT-2, T-2 tetraol, Roridan A, verrucarin A, and verrucarol) also inhibited mitochondrial protein synthesis. Both chloramphenicol and T-2 decreased the label found in electrophoretic bands corresponding to mitochondrial translation products. These results are significant because cytoplasmic and mitochondrial systems of protein synthesis are different. This was the first time that T-2 has been shown to affect a prokaryotic-like system.

In an effort to correlate the results seen in cell systems with whole-animal lethality, studies have begun on the in vivo incorporation of labeled amino acids into the trichloroacetic acid (TCA)-precipitable fraction of livers from control and T-2-exposed rats. Eight hours after giving rats twice a normal LD_{50} dose, the intoxicated rats had about twice the radioactivity associated with the TCA-precipitable fraction compared to the controls. Additional time periods during the course of the intoxication will be observed to see if, at any time, there is a suppression of amino acids uptake which would be an indication of protein synthesis inhibition in whole animals.

PRESENTATIONS:

1. **Kemppainen, B. W., R. T. Riley, J. G. Pace, F. J. Hoerr, and J. L. Joyave.** Effects of DMSO on the penetration of T-2 toxin through excised human and monkey skin. Presented, 8th World Congress on Animal, Plant and Microbial Toxins, IST, Newcastle upon Tyne, England. 13 August 1985.

2. **O'Brien, J., W. Thompson, and J. Pace.** T-2 toxin: effects and metabolism in vero cells and rat hepatocytes. Presented, FASEB, Anaheim, CA. April 1985.

3. **Pace, J. G., M. R. Watts, and W. J. Canterbury.** T-2 toxin-induced inhibition of mitochondrial protein synthesis. Presented, FASEB, Anaheim, CA. 25 April 1985.

4. **Pace, J. G., W. J. Canterbury, and C. Matson.** (1) T-2 toxin-induced inhibition of mitochondrial protein synthesis, and (2) Fate and distribution of [^{3}H]T-2 toxin in topically exposed guinea pigs. Presented, Colby-Sawyer College, Gordon Conference, New London, NH. 19 June 1985. (2 presentations, chair session).

5. **Pace, J. G., M. R. Watts, E. Burrows, and C. Matson.** Identification of metabolites of T-2 toxin in guinea pigs. Presented, 8th World Congress on Animal, Plant and Microbial Toxins. IST, Newcastle upon Tyne, England. 13 August 1985.

6. **Thompson, W. L., J. C. O'Brien, and R. W. Wannemacher, Jr.** Variable toxicity of trichothecene mycotoxins in cell culture systems. Poster presentation, Society of Toxicology meetings, March 1985, San Diego, CA.

7. **Thompson, W. L., J. G. Pace, R. W. Wannemacher, Jr.** The effects of T-2 mycotoxin on cultured heart cells from chicken embryos. Presented, FASEB, Anaheim, CA. April 1985.

PUBLICATIONS:

1. **Kemppainen, B. W., R. T. Riley, and J. G. Pace.** 1984. Penetration of [^{3}H]T-2 toxin through excised human and guinea pig skin during exposure of [^{3}H]T-2 toxin adsorbed to corn dust. *Food Chem. Toxicol.* 22:893-896.

2. **Kemppainen, B. W., R. T. Riley, and J. G. Pace.** 1985. Penetration of mycotoxins through excised human skin. *In* R. Bronaugh and H. Maibach (ed.), Percutaneous penetration, Chapter 33. Marcel Dekker, Inc., N.Y., (In Press).

3. **Kemppainen, B. W., R. T. Riley, J. G. Pace, and F. J. Hoerr.** 1985. The effects of skin storage conditions and concentration of applied dose of [^{3}H]trichothecene on the penetration of T-2 toxin through excised human and monkey skin. *Food Chem. Toxicol.* (In Press).

4. **Kemppainen, B. W., R. T. Riley, J. G. Pace, F. J. Hoerr, and J. L. Joyave.** 1985. Effects of DMSO on the penetration of T-2 toxin through excised human and monkey skin. *Toxicon* 23:4.

5. **Neufeld, H. A., J. G. Pace, and R. W. Hutchinson.** 1985. Detection of microorganisms by bio- and chemiluminescent techniques, pp.51-65. *In* W. Nelson (ed.), Rapid detection and identification of microorganisms. YCH Publishers, Inc., N.Y.

6. **O'Brien, J., W. Thompson, and J. Pace.** 1985. T-2 toxin: effects and metabolism in vero cells and rat hepatocytes. *Fed. Proc.* 44:1038.

7. **Pace, J. G., M. R. Watts, E. P. Burrows, R. E. Dinterman, C. Matson, E. C. Hauer, and R. W. Wannemacher, Jr.** 1985. Fate and distribution of ^{3}H-labeled T-2 mycotoxin in guinea pigs. *Tox. Appl. Pharmacol.* 80(In Press).

8. **Pace, J. G., M. R. Watts, E. Burrows, and C. Matson.** 1985. Identification of metabolites of T-2 toxin in guinea pigs. *Toxicon* 23:4.

9. **Pace, J. G., M. R. Watts, and W. J. Canterbury.** 1985. T-2 toxin-induced inhibition of mitochondrial protein synthesis. *Fed. Proc.* 44:1802.

10. **Thompson, W. L., and R. W. Wannemacher, Jr.** 1985. Detection and quantitation of T-2 mycotoxin with a simplified protein synthesis inhibition assay. *Appl. Environ. Microbiol.* 48: 1176-1180.

11. **Thompson, W. L., J. C. O'Brien, and R. W. Wannemacher, Jr.** 1985. Variable toxicity of trichothecene mycotoxins in cell culture systems. *The Toxicologist.* 5:211.

12. **Thompson, W. L., J. G. Pace, R. W. Wannemacher, Jr.** 1985. The effects of T-2 mycotoxin on cultured heart cells from chicken embryos. *Fed. Proc.* 44:537.

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1. **Mirocha, C. J., R. A. Pawlosky, K. Chatterjee, S. Watson, W. Hayes.** 1983. Analysis for *Fusarium* toxins in various samples implicated in biological warfare in Southeast Asia. *J. Assoc. Off. Anal. Chem.* 66:1485-1499.

2. **Uneo, Y.** 1980. Trichothecene mycotoxins: mycology, chemistry, and toxicology. *Adv. Nutr. Res.* 3:301-353.

3. **Bessey, O.** 1976. Status of marine biomedical research. *Environ. Health Prospct.* 13:147-163.

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5. **Bates, H. A., and H. Rapoport.** 1975. A chemical assay for saxitoxin, the paralytic shellfish poison. *J. Agr. Food Chem.* 23:237-239.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA305922	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 12 26	H.TERM	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS12	AF	005
b. CONTRIBUTING				
c. CONTRIBUTING	DA LRRDAP, FY86- 01			

11. TITLE (Precede with Security Classification Code) Pathophysiology of Low Molecular Weight Mammalian Peptides

12. SUBJECT AREAS
0605 Clinical Medicine; 1503 Defense; 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 12	85 10	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	1.0	159
c. TYPE	d. AMOUNT	86	0.0	0
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathophysiology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Saviolakis, G A
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7181
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Bunner, D L
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Therapy; (U) Mammalian Peptides; (U) Neurotransmitters; (U) Leukotrienes; (U) RAD I; (U)Lab Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To study the basic mechanisms of action and their physiological effects on host vital systems of mammalian low molecular weight peptides, such as neurohormones, known and putative peptide neurotransmitters, and leukokines. To develop therapeutic interventions.

24. (U) Establishment of cell culture systems, representative of neural tissue, muscle, and liver, for: (1) characterization of peptide and non-peptide receptors; (2) studies of receptor regulation; (3) evaluation of intra-cellular events; (4) studies of interactions between peptide and non-peptide transmitters; and (5) evaluation of putative antagonists and drugs.

25. (U) 8412-8509-A human neural cell line was used to study receptors for insulin (a neurohormone as well as a peripheral hormone) and cholinergic neurotransmitters. Specific insulin receptors (IR) were found on these cells, whose physicochemical characteristics were similar to receptors in non-neural tissues. IR could be down-regulated by insulin and cholinergic antagonists. Regulation by interleukin-1 (a putative neuropeptide) was inconsistent. IR antibody prevented insulin binding. Cholinergic receptors of the muscarinic type were also found and are being characterized. Primary hepatocytes and continuous liver and muscle cell lines were used to study the effects of interleukin-1 (IL-1) on protein metabolism. Culture conditions were evaluated thoroughly. IL-1 gave consistent results only on myocytes. (B) Studies to evaluate the pharmacokinetics and effects of intranasal insulin were initiated using a hydrophobic vehicle (fusidic acid).

Continued under DAOG 1526.

BODY OF REPORT

PROJECT NO. 3M161102BS12: Science Base/Medical Defense Against BW

WORK UNIT NO. S12-AF-005: Pathophysiology of Low Molecular Weight Mammalian Peptides

PRINCIPAL INVESTIGATOR: Saviolakis, G. A., LTC

ASSOCIATE INVESTIGATOR: Bunner, D. L., COL, M.D.

Background:

Small molecular weight endogenous peptides, such as neuropeptides and monokines, have major effects on physiological control of vital organ systems, such as cardio-respiratory, vascular, endocrine, and immune systems, and on behavior (1). Due to their demonstrated potency, many of these peptides may be important as potential BW agents. The purposes, therefore, of this work unit are: (a) to assess the pathophysiological effects of selected groups of peptides; (b) to develop methods for their detection; and (c) to develop therapeutic interventions to mitigate their effects on military personnel.

Summary:

Research efforts during the past year had three primary elements:

(A) Evaluation of in vitro systems to study the basic mechanism of action of select neuropeptides, neurotransmitters, and monokines. These included: (A_1) Application of an established human neural cell line, the Y-79 retinoblastoma cell, to studies of insulin receptors and responses; (A_2) Application of the same cell line to studies of cholinergic receptors; and (A_3) Use of continuous or primary hepatocyte and muscle cell lines to evaluate the catabolic effects of interleukin-1.

(B) Development of an in vivo system to study a limited aspect of peptide pharmacokinetics, namely absorption through respiratory mucosae. This utilizes recently developed hydrophobic vehicles (fusidic acid; bile salts) for intranasal insulin therapy.

(C) Definition of the scope and content of this work unit through discussions and consultations with USAMRIID and other DOD investigators from WRAIR, USAMRICD, NMRI, and USUHS. A meeting was held at USAMRIID on August 6, 1985, and, as a result, programmatic plans are currently being completed.

Progress:

(A_1) - The human Y-79 retinoblastoma cell line is easily grown in suspension culture and can be induced to differentiate into neuronal and glial cells. Specific insulin receptors were demonstrated on these cells. These receptors are kinetically similar to ones present on non-neural cells, except for the absence of negative co-operative interactions among them. They are down-regulated by insulin and cholinergic antagonists. Interleukin-1, which is produced by astrocytes in the central nervous system (CNS), had inconsistent effects on insulin receptor regulation. Except for down-regulation by insulin, other insulin effects, such as glucose and amino acid transport, and decrease in catecholamine-stimulated cyclic AMP levels, were not found. Antibodies against the insulin receptor inhibited insulin binding.

(A_2) - Cholinergic receptors of the muscarinic type (the predominant type in CNS), but not of the nicotinic type, were also found on Y-79 cells. Progress in characterizing these receptors further and in studying their regulation has been impeded by technical problems (mycoplasma contamination, high endotoxin levels in a serum lot) which were only recently overcome.

Cumulative results and experience with this cell line suggest its utility in neuropeptide and neurotransmitter receptor studies, in studies of interactions among peptide and non-peptide transmitters, and, possibly, in screening for neurotoxins. The effect of insulin anti-receptor antibody suggests its use in mitigating the adverse effects of hybrid insulin/toxin peptides.

(A_3) - In response to interleukin-1, protein synthesis and degradation have been studied in three cultured cell lines: the continuous clone 9 rat hepatocytes, primary rat hepatocytes, and mouse L6 muscle cells. Significant effort was expended in standardizing culture conditions, as cell preparation and age, incubation conditions, and serum effects are all factors contributing to substantial inter-assay variation and inconsistent results. In general, L6 myocytes gave the more consistent results to fetal bovine serum (FBS) (decreased protein degradation, increased synthesis), dexamethasone (DXM) (increased degradation, decreased synthesis), and to interleukin-1 (IL-1) (increased degradation and synthesis). Both clone 9 and primary hepatocytes were much less consistent in response to IL-1. Their responses to FBS and DXM, even though consistent, had high inter-assay variations. These findings emphasize the need for careful extrapolation of in vitro results to the in vivo situation and the necessity for in vivo verification of findings as they arise. In addition, currently commercially available IL-1, diluted in FBS, is not appropriate for this type of study, due to potent independent effects of FBS. We have reason to believe that IL-1 diluted in human serum albumin, soon to be available, may alleviate this problem.

Two other cell culture lines, the lymphoma LBRM-33-5A cells and human foreskin fibroblasts, widely used for bioassay of IL-1 activity, were also adopted in the laboratory, and their growth responses to IL-1 are being evaluated.

(B) - Bile salts (2) and fusidic acid (3) were recently shown to facilitate intranasal absorption of insulin. Although only 10% or less of the administered hormone was absorbed, a biological effect (ie., decrease in blood glucose level) was observed when 50 U/0.1 ml insulin was given to human subjects 2, 3. Studies exploring this system were performed in collaboration with Dr. Donald Creasia of the Pathophysiology Division. In anesthetized rats, insulin (1-2 units/kg) in 1% (v/v) fusidic acid lowered blood glucose by 40% and 60% after intranasal or intratracheal instillation, respectively. With an equivalent dose of ^{125}I-labeled insulin, less than 5% of the radioactivity was detected in the blood of which only 23% was acid-precipitable. These are still very preliminary results but the potential of this route of peptide administration will be further explored for evaluating toxicological risk and therapeutic benefits.

(C) - After several discussions with other DOD (WRAIR, USAMRICD, NMRI, USUHS) investigators, a round-table discussion was held at USAMRIID on August 6, 1985 to help focus the scope and research effort on work with regulatory peptides. Meetings to define the workscope are currently held at USAMRIID and the program will soon be implemented.

PRESENTATIONS:

1. **Saviolakis, G. A., A. P. Kyritsis, and G. J. Chader.** Specific insulin receptors in human Y-79 retinoblastoma cells. Presented at the Annual Meeting of the Association for Research in Vision and Ophthalmology, Sarasota, FL, May 5-10, 1985.

PUBLICATIONS:

1. **Saviolakis, G. A., A. P. Kyritsis, and G. J. Chader.** 1985. Specific insulin receptors in human Y-79 retinoblastoma cells. *Invest. Ophthalm.* (ARVO Abstracts) 11:126.

LITERATURE CITED

1. **Krieger, D. T.** 1984. Brain Peptides. *Vit. Horm.* 41:1-50.

2. **Moses, A. C., G. S. Cordon, M. C. Carey, and J. S. Flier.** 1983. Insulin administered intranasally as an insulin-bile salt aerosol: effectiveness and reproducibility in normal and diabetic subjects. *Diabetes.* 32:1040-1047.

3. **Silver, R., A. C. Moses, M. C. Carey, G. Gordon, and J. S. Flier.** 1985. Derivatives of fusidic acid: novel adjuvants for transnasal peptide absorption. *Clin. Res.* 33:288A.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 302657	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763A	3M263763D807	AA	011
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82-83-6.2/3			

11. TITLE (Precede with Security Classification Code) (U) Exploratory Development of Vaccines Against Anthrax

12. SUBJECT AREAS 1503 Defense; (U) 0613 Microbiology; (U) 0620 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	0.7	68
c. TYPE	d. AMOUNT	86		
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Bacteriology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Leppla, S H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Ezzell, J W
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) BW Defense; (U) Microbiology; (U) Anthrax; (U) Vaccines; (U) Lab Animals; (U) Guinea Pigs; (U) RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Since anthrax is considered to have significant potential as a biological warfare agent, it is essential that the following objectives be undertaken in order to protect at-risk troops: (a) identify effective and safe antigens, antigen mixtures, or live attenuated strains which confer protection against anthrax infection; (b) develop procedures for large scale vaccine production.

24. (U) Test ability of purified anthrax toxin protein components, purified polysaccharides, attenuated spore vaccines, and protein-polysaccharide conjugates to protect against aerosol exposure; produce vaccine components on large scale from cloned genes; develop improved adjuvants or vaccine delivery modes to enhance immune response.

25. (U) 8410-8509-Tangential flow filtration was developed as a safer method of harvesting the 50-L anthrax fermenter cultures. Hydroxylapatite was shown to be useful for the adsorption of anthrax toxin lethal factor (LF) and edema factor (EF) from *B. anthracis* culture supernatant. A fraction of the PA was identified which has increased negative charge and decreased potency; this was separated from fully potent PA. PA was cleaved by mild trypsin treatment into two fragments; these were purified for testing as vaccines. Purified toxin was supplied to eight different contractors and university collaborators. A single dose of 10^6 Sterne spores was shown to provide some protection against challenge with virulent anthrax. Cell fusions were performed and additional hybridomas producing monoclonal antibodies to PA and EF were obtained. Peripheral white cells from recently boosted laboratory personnel were provided to investigators at WRAIR, who successfully produced human hybridoma antibodies to PA.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. D807-AA-011: Exploratory Development of Vaccines Against Anthrax

PRINCIPAL INVESTIGATOR: Stephen H. Leppla, Ph.D.

ASSOCIATE INVESTIGATORS: John W. Ezzell, Ph.D.
Stephen F. Little, Ph.D.
Gregory B. Knudson, Ph.D.

Background:

The possibility that *Bacillus anthracis* might be used as a BW agent against U.S. forces requires that a vigorous effort be made to develop and maintain modern, effective methods to prevent, diagnose, and treat anthrax infection. The current human anthrax vaccine was developed more than 20 years ago. It consists of the culture supernatant of the toxigenic Sterne strain. This vaccine is expensive, reactogenic, and appears to have limited immunogenicity, as indicated by the need for six initial immunizations, followed by yearly boosters. Efforts over several years have sought to improve this chemical vaccine through optimization of fermenter and recovery techniques so as to increase yields and purity of the protective antigen (PA) component. More recently, consideration has been given to using the cloned PA gene for production of the vaccine protein or its genetically altered variants in heterologous bacterial expression systems.

Since vaccine tests in humans must be limited to measures of antibody response, vaccine design has relied on the results of animal trials. Reports from the 1950's and 60's indicate that immunization with PA vaccines may not provide protection against all virulent strains of *B. anthracis*. These reports have been confirmed in studies reported in the USAMRIID Annual Report, 1984.

For the reasons stated above, consideration has also been given to use of the live Sterne strain as a human vaccine. This toxigenic, but noncapsulated strain was developed more than 50 years ago and has seen wide and successful use in protection of livestock. Furthermore, incomplete reports show that a Sterne-type strain (STI) was used in several million persons in the Soviet Union with apparent efficacy. Anecdotal reports of severe reactions or infections following STI vaccination cannot be evaluated from the available literature. To be acceptable for use in the United States, it is preferable that the Sterne strain be further attenuated through genetic manipulation.

Consideration is also being given to the use of monoclonal antibodies in diagnostic techniques or in treatment of infections. An ongoing effort to develop mouse and human monoclonal antibodies is occurring through in-house, collaborative, and contract work.

Summary:

During this period, efforts to improve the production of the vaccine components of anthrax toxin focused on recovery of proteins from the culture supernatant. Tangential flow filtration was put into use as a safer way to remove bacteria from

the culture. Hydroxylapatite was found to be an efficient batch adsorbant for recovery of lethal factor (LF) and edema factor (EF). High-resolution chromatographic and electrophoretic methods revealed that the purified protective antigen (PA) contained a species with slightly increased negative charge. This species was much less toxic than the major PA fraction. Purified toxin components were supplied on demand to investigators at USAMRIID and elsewhere.

Mild trypsin treatment produced two large fragments from PA. Conditions were found for purifying these fragments and keeping them soluble, to facilitate their use in functional assays.

Evaluation of the possible use of the Sterne strain as a vaccine continued with a test of the minimum immunization schedule which confers protection. A single dose of 10^6 or two doses of 10^5 spores of Sterne provided significant protection against challenge with Vollum 1b strain.

Additional fusions were performed and approximately 24 new hybridomas for PA were obtained. A collaborative effort with WRAIR led to production of candidate human hybridomas to PA.

Progress:

The 50-liter fermenter continues to be used successfully to produce *Bacillus anthracis* toxin components. Following optimization of growth medium, attention has been focused on improving the procedures for recovery of toxin from the culture supernatant. Hydroxylapatite is especially effective for adsorbing LF and EF from undiluted culture supernatants. A limitation, however, in the use of hydroxylapatite, is its incompatibility with EDTA, the most effective inhibitor of the zinc-dependent protease produced by *B. anthracis*. This limitation has now been overcome through a systematic comparison of zinc chelating agents. The chemical, 1,10- phenanthroline, has been found to be a very effective protease inhibitor; but it does not have the property of dissolving the hydroxylapatite or inhibiting protein adsorption, as does EDTA.

The development of reliable systems for the production of anthrax toxin has allowed attention to be turned to a detailed examination of the toxin proteins. Techniques developed to examine the purified proteins include high-resolution, anion exchange chromatography employing Mono-Q columns (Pharmacia); and electrophoresis on nondenaturing polyacrylamide slab gels. When examined by these procedures, PA preparations contained several species differing slightly in charge. These species could be produced on a semi-preparative scale by using the mono-Q columns. The fraction with increased negative charge was shown in the macrophage toxicity assay to be at least 10-fold less potent. This result may explain indications that preparations of toxins produced several years ago were less potent. Availability of the electrophoretic and the HPLC methods for analysis of PA preparations should make routine quality control of preparations possible and assure the production of highly potent preparations. By using the methods described in previous annual reports, we have maintained stocks of the three individual toxin components at adequate levels so that requests from USAMRIID investigators have been promptly filled. In addition, during 1985, purified toxin components have been provided to eight different contractors and collaborators outside USAMRIID.

An important step has been made in analysis of the structure-function relationships in the PA protein through pursuit of an observation made by a collaborator, Dr. David McKay of the University of Colorado. Dr. McKay noted that very mild trypsin treatment of PA caused a single cleavage, resulting in production of two fragments of approximately 60 and 20 kilodaltons (Kd). These fragments remained associated until the protein was either heated to 45°C or exposed to strong denaturants. Heating the nicked toxin to 45°C caused separation of the fragments, demonstrated by size-exclusion HPLC. However, the 60-Kd fragment produced in this way tended to precipitate under most conditions. An alternative procedure for seperating the fragments has been found which allows separation of the two fragments in a non-denatured, soluble form. To accomplish this, the nicked toxin was applied directly to a Mono-Q column equilibrated at pH 9. Application of a salt gradient led to elution, first of the 20- and then of the 60-Kd fragment. These can be obtained and maintained in solution by dialysis at pH 9. These purified fragments are now available for testing, both as candidate vaccines and as reagents to examine the binding and internalization of PA on eucaryotic target cells. Such studies should localize the receptor-binding domain of PA to one of these two fragments.

Animal trials to evaluate candidate vaccines are focusing on comparison of the chemical PA vaccine and the live Sterne vaccine. As previously reported, the PA vaccine does not provide complete protection against 9 of 25, *B. anthracis*, challenge strains. Strains overcoming PA vaccination have been termed "vaccine-resistant" isolates. The NH and Ames strains are examples. The possibility that this phenomenon might be due to differences in the virulence of vaccine-resistant-strain spores was examined by measuring LD_{50}'s of several strains. LD_{50} values for im challenge were approximately 30 - 50 CFU for Vollum, NH and Ames; but were 400 CFU for Vollum 1b. The apparent loss in virulence of Vollum 1b (used in some of the experiments which defined the vaccine-resistant strains) may necessitate the reexamination of this phenomenon. British investigators have confirmed that Ames and NH challenge can overcome immunization with chemical vaccines. Future plans include analysis of whether the vaccine-resistance phenomenon is observed when PA-immunized animals are challenged by the aerosol route.

In comparisons of the efficacy of chemical and live vaccines, it is also necessary to consider the optimization of immunization schedules. Previous experiments have, in general, tested two doses of both types of vaccines, spaced at two-week intervals, followed by challenge two weeks after the last dose. In order to determine whether a single immunization with Sterne spores provides protection, an experiment was performed (Table 1). Graded doses of Sterne spores were given either as a single immunization or as two immunizations spaced at an interval of two weeks. Intramuscular challenge with 2500 spores was performed two weeks after the last immunization. The data indicate that a single dose of 10^6 spores, or of two doses of 10^5 spores, were necessary to induce a detectable ELISA titer to PA and to induce some degree of protection against challenge.

Another research effort contributing directly to anthrax vaccine improvement is the continuing isolation of hybridomas and production of monoclonal antibodies to anthrax toxin components. Under a contract established with Hazelton Laboratories, a fusion was performed with PA-immunized mice. Performance of the fusion and coordination with the contractor were performed successfully, but only two useful monoclones resulted. Subsequently, additional fusions to produce PA monoclonal antibodies were performed by the Virology Division hybridoma group in December 1984 and April 1985. Each fusion yielded approximately twelve clones which appeared valuable. Recloning and analysis of these hybridomas are continuing.

An Edema Factor (EF) fusion performed in June 1984 was successfully completed with the selection of 15 candidate hybridomas. Unfortunately these clones proved to be very unstable when placed into mice for ascitic fluid production. Only one clone proved stable upon repeated subcloning. This clone has been expanded and sent to Salk Institute for ascitic fluid production. The continuing difficulty in producing monoclonal antibodies to EF may reflect the interaction of this protein with the eucaryotic protein, calmodulin, and the possible perturbation of lymphocyte metabolism expected if EF were internalized. In future attempts to produce EF monoclones it may be useful to chemically or proteolytically inactivate the adenylate cyclase activity of the protein.

One immediate application of the monoclonal antibodies described above is their use in immunoabsorption chromatography for purification of toxin components. An ongoing contract with Dr. Joseph Burnett, University of Maryland School of Medicine, has this goal. Monoclonal antibodies, crude toxin supernatant fluids, and purified LF have been supplied to Dr. Burnett as needed to support his work.

In other studies employing monoclonal antibodies, USAMRIID investigators have supported the efforts of COL Jerald Sadoff, WRAIR, to produce human monoclonal antibodies to PA. Peripheral blood was collected from USAMRIID personnel three to four days following booster immunization with anthrax vaccine, and the resulting cells were fused to produce hybridomas. Large numbers of positive clones were obtained by COL Sadoff. These have been cloned repeatedly and will now be expanded to obtain antibodies. It is anticipated that USAMRIID Investigators will be able to identify neutralizing monoclonal antibodies from among this large collection.

PRESENTATIONS: None

PUBLICATIONS:

1. **Allured, V. S., L. M. Case, S. H. Leppla, and D. B. McKay.** 1985. Crystallization of the protective antigen protein of *Bacillus anthracis*. *J. Biol. Chem.* 260:5012-5013.

2. **Wade, B. H., G. G. Wright, E. L. Hewlett, S. H. Leppla, and G. L. Mandell.** 1985. Anthrax toxin components stimulate chemotaxis of human polymorphonuclear neutrophils. *Proc. Soc. Exper. Biol. Med.* 179:159-162.

Table 1. Effect of immunization schedule on protection of guinea pigs from challenge[a]

Immunization Schedule		Response	
No. Doses	Spores/dose	Anti-PA Antibody	Survival after Challenge (%)
1	10^4	14[b]	50[c]
1	10^5	29	50
1	10^6	260	70
2	10^4	36	44
2	10^5	230	80
2	10^6	5600	90

[a]Guinea pigs were immunized im with Sterne spores.
[b]Average reciprocal ELISA titer at time of challenge.
[c]Challenge with 2500 Vollum 1b spores occurred 2 weeks after the last immunization.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 302660	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	263763	3M263763D807	AB	012
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

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83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	2.0	242
c. TYPE	d. AMOUNT	86		
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Airborne Diseases Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Stephenson, E H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Williams, J C
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Snyder, C E, Jr

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Q Fever; U) Coxiella burnetii; (U) Vaccines

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) *Coxiella burnetii* is perceived to have significant potential as a BW agent. The currently available vaccine is reasonably protective, but highly reactogenic. An efficacious yet more safe vaccine needs to be developed and stockpiled to protect at-risk US troops. The objective is to proceed with evaluation of a chloroform-methanol extracted residue (CMR) vaccine to assess feasibility for use in humans. Simultaneously a new generation subunit vaccine is being sought, which can be produced readily without the requirement for high containment laboratories.

24. (U) Determine toxicity, safety, efficacy, and dose response to CMR vaccine. Extend animal model testing of CMR to identify and quantify humoral and cell-mediated immune responses. Proceed with human use evaluations if safety and efficacy are demonstrated. Define immunogenic subunits of *C. burnetii* to provide bases for development of a subunit vaccine.

25. (U) 8410-8509 - Time- and dosage-dependent lymphocyte unresponsiveness in C57/BL/10 ScN mice after vaccination with phase I whole cell (WC) vaccine correlated with appearance of hepatomegaly, splenomegaly, and death. CMR vaccine did not induce immunopathologic reactions. Induction of immunosuppression and negative regulation in mice by phase I WC vaccine is antigen-specific. Observations suggest that immunologic unresponsiveness can be genetically unlinked from the gross pathologic responses. Lymphocyte unresponsiveness in humans with Q fever endocarditis apparently is modulated by an antigen-specific T suppressor cell that secretes a lymphokine to stimulate PGE_2 production by adherent cells. Definite ultrastructure differences based on variations in chemical composition exist between LPS of phase I and phase II cells. A specific immune response was induced in rabbits and mice with a 29.5K dalton, protein-containing fraction of *C. burnetii*. Monoclonal antibodies have been produced to this fraction.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO: 807-AB-012: Exploratory Development of Vaccines Against Q Fever

PRINCIPAL INVESTIGATOR: E. H. Stephenson, D.V.M., Ph.D., COL, VC

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Background:

Infection of individuals with phase I *Coxiella burnetii*, the etiologic agent of Q fever, results in the development of serum antibodies and cell-mediated immunity. These are readily detected by various in vivo and in vitro assays. Protection against natural or experimentally induced infection is obtained by vaccination with suspensions of killed phase I whole cell (WC) vaccines; however, these vaccines induce immunopathologic reactions in humans and animals. Previous studies on the safety and efficacy of WC vaccines have centered around a determination of acceptable dosage of vaccine based on side effects, on antibody conversion rate of vaccinees, and on the detection of cell-mediated immunity with a delayed-type skin test or in vitro lymphocyte proliferation assay. Adverse reactions obtained during the vaccine trials have prevented widespread and unconditional use of the phase I WC vaccines. Attempts to remove components that induce adverse reactions have met with limited success because partially purified phase I antigens obtained by various organic or aqueous solvent extractions were effective immunogens only when used with adjuvants. Thus, the relationship between phase I WC vaccine and those immunogenic fractions that induce deleterious tissue reactions, delayed-type hypersensitivity, in vitro lymphocyte proliferation, and resistance to infection is not known.

Summary:

Major advancements were made in six areas of the research programs within the work unit.

1) Time- and dosage-dependent lymphocyte unresponsiveness occurred in C57BL/10 ScN mice after immunization with phase I WC vaccine of *C. burnetii*, and the response correlated with the appearance of hepatomegaly, splenomegaly, and death. Concurrently, generalized non-specific splenic and lymph node lymphocyte hyporesponsiveness was developed to concanavalin A(ConA), phytohemagglutinin-P(PHA), and pokeweed mitogens. Chloroform-methanol residue (CMR) vaccine prepared from phase I WC, by contrast, did not induce immunopathologic reactions or suppression of mitogenic and antigenic lymphocyte proliferation. Lymphocyte responsiveness after injection of CMR vaccine correlated with protective efficacy. Although some components of phase I *C. burnetii* are important determinents of the immunopathologic reactions, adverse responses are not solely due to phase I lipopolysaccharide (LPS). The reactivity of CMR can be restored readily by addition of the chloroform-

methanol extract (CME). Apparently CMR contains suppression-inducing determinants, while CME contains specific or non-specific components. The active component in CMR seems to be a dithiothreitol (DTT)-soluble fraction with a molecular size of less than 3,500 daltons.

2) Experimental data from experiments with prostaglandin synthesis inhibitors, macrophage toxins, cell-depletion techniques, and culture protocols suggest that the induction of suppression and negative regulation of mouse spleen cells by phase I WC vaccine is antigen-specific, and that the mechanism of action is non-specific with several cell types being involved.

3) Following phase I *C. burnetii* infection or vaccination, splenic lymphocytes were suppressed in the absence of splenomegaly in BALB/c mice and congenic strains. These observations strongly suggest that immunologic unresponseiveness can be genetically unlinked from the gross pathologic responses. Other data indicate that the Igh-controlled functional specificities may govern, at least in part, the in vivo development of splenomegaly.

4) Suppression of lymphocyte responsiveness by *Coxiella* antigens in humans was in part mediated by an antigen-nonspecific, glass-adherent cell. The failure of T-cells and $T8^+$ (suppressor)-depleted cells to respond to *Coxiella* antigens suggests additional mechanisms of suppression are operative. Further, we have shown that the adherent suppressor cells work via prostaglandin E_2 (PGE_2) production. Lymphocyte unresponsiveness in humans with Q fever endocarditis apparently is modulated partly by an antigen-specific T suppressor cell which secretes a lymphokine to stimulate PGE_2 production by adherent cells.

5) Definite ultrastructural differences were observed between phase I LPS and phase II LPS. The differences undoubtedly are based on variations in the chemical composition of each. Phase I LPS, but not phase II LPS, contain unknown neutral sugars: galactosaminuronic acid and compound X. All other components were identical in the LPS of both phases. The low or undetectable amounts of fatty acids in *C. burnetii* LPS differentiate this non-toxic LPS from gram-negative bacterial LPS. Compound X found in phase I LPS was shown to be galactosaminuronyl glucosamine. On the basis of SDS-PAGE electrophoretic patterns for LPS, the 9 Mile II and M44 strains had single low molecular weight bands; 9 Mile, Ohio, and Henzerling strains contained no LPS II; K and Australian strains had a mixture of LPS I and LPS II; and the P strain contained a small amount of LPS II in addition to LPS I.

6) A specific immune response was induced with the 29.5K dalton protein-containing fraction of *C. burnetii* in rabbits and mice. Of 24 monoclonal antibody-producing cell lines obtained, 8 are being expanded for use in future studies. Two-dimensional electrophoresis was used to compare the proteins extracted from several strains of *C. burnetii*. Although great similarity existed among the different strains, a number of specific differences allowed comparisons to be made. Also, preliminary studies showed the existence of 1 to 4 DNA-binding proteins in each of 5 *C. burnetii* strains.

Progress:

Immunomodulation in Mice Recent studies have shown that immunization of C57BL/10 ScN, endotoxin-nonresponder mice with phase I WC vaccine of *C. burnetii* induced immunopathologic reactions. Time- and dosage-dependent lymphocyte unresponsiveness followed immunization, and correlated with the appearance of hepatomegaly, splenomegaly, and death of mice. Concurrently, generalized non-specific splenic and lymph node lymphocyte hyporesponsiveness was developed to concanavalin A, phytohemagglutinin-P, and pokeweed mitogens. Splenic and lymph node lymphocytes from immunized mice were down-regulated below normal control levels by specific recall antigen. In these studies, cellular expression of negative modulation was a property of phase I of *C. burnetii* since phase II cells were not effective in the induction of lymphocyte unresponsiveness. Components of the phase I WC vaccine that were not required for protective efficacy were successfully extracted with chloroform-methanol. We have shown that a chloroform-methanol residue (CMR) vaccine prepared from phase I WC does not induce immunopathologic reactions or suppression of mitogenic and antigenic lymphocyte proliferation after i.p. injections in C57BL/10 ScN mice. The ability of CMR vaccine to induce in vitro lymphocyte proliferative responsiveness correlated with the protective efficacy of this delipidated vaccine. Furthermore, deleterious tissue reactions and suppression of mitogenic and antigenic lymphocyte responsiveness occurred when CMR vaccine was reconstituted with CM extract (CME). This suggests that virulence factors of *C. burnetii* fractionated into both the CME and the phase I CMR are required for induction of the adverse reactions. Induction of immunoregulatory mechanisms to function in pathologic and suppressor modes may be initiated by the composition of the antigenic mass. Unique determinants present on phase I *C. burnetii* might induce specific suppressor cells that regulate the activity of lymphocytes to mitogens and antigens. No unique epitopes of *C. burnetii* have been identified which induce this immunological unresponsiveness. We have shown that components associated with phase I *C. burnetii* induce immunologic hyporesponsiveness and negative modulation of lymphocytes to mitogens and recall antigens. These components are easily separated from phase I *C. burnetii* by chloroform-methanol extraction which yields an efficacious CMR vaccine. The CMR vaccine contains many proteins and phase I lipopoly-saccharide (LPSI). Although LPSI is considered to be a major determinant of virulence expression and infection, the presence of LPSI in CMR vaccine does not induce the deleterious reactions and suppression of in vitro lymphocyte proliferation. In a previous study, phase I WC vaccine, but not phase II WC vaccine, induced pathologic reactions, including suppression of in vitro lymphocyte proliferation. It appears, therefore, that some components of phase I *C. burnetii* are important determinants of immunopathologic reactions; however, adverse reactions cannot be assigned solely to LPSI. The importance of LPSI in the development of immunity has been verified in experimental animal models which require development of antibody and cellular responses to LPSI for protection against phase I challenge.

Neither viable phase I *C. burnetii* nor killed phase I WC vaccines are readily cleared by the host, and significant splenomegaly persists up to 35 days after injection of mice. During this time, viable organisms or WC vaccine can be detected in spleen impression smears, yet CMR vaccine antigen was cleared from the spleen. Reconstitution of CMR vaccine with CME restored the refractory nature of the CMR vaccine to that of the phase I WC vaccine. These results indicate that phase I CMR vaccine contains suppression-inducing determinants while CME contains specific or non-specific components. Studies were initiated to fractionate the CMR vaccine and CME so that the interacting molecules could be purified and characterized as to their immunogenicity and pathogenicity.

We were successful in the fractionation of the CMR vaccine into soluble components which participate in the suppressive complex of *C. burnetii*. Although many different chemical and enzymatic treatments were tested for the inactivation of the biological activity of the suppressive complex, the extraction of CMR vaccine with DTT was the most effective treatment for the preparation of an aqueous soluble component. The DTT-solubilized (DTT-S) components, when lyophilized and reconstituted with CME, were effective in eliciting the immunopathologic reactions of the phase I WC vaccine. A component in the DTT-S fraction with a molecular size of less than 3,500 daltons appears to be the active compound.

Mechanisms of Immunomodulation The mechanism through which phase I *C. burnetii* WC vaccine suppresses in vitro lymphocyte proliferation is not known. Viability studies of normal spleen cell cultures treated with 5 μg of WC vaccine per ml suggest that reduced responsiveness was not attributable to overt toxicity, but rather that the condition results from a more subtle or indirect action on the part of the inducing agent. Several different mechanisms of immune suppression are known to influence lymphocyte proliferation responses. Suppression due to thymus-derived lymphocytes is well established. Previous studies have shown that injection of WC vaccine into mice results in proliferation in situ of a particular population of lymphoid cells in the spleen. Although the identity of the spleen cells proliferating in response to WC vaccine was not determined, it is possible that the cells could be assigned T-suppressor cell activity. Alternatively, the observed response to WC vaccine may possibly result from modulation by macrophages. Macrophages are known to play a key role in the regulation of lymphocytic proliferation and to mediate immunosuppression during numerous infectious diseases. Previous studies have shown that phase I *C. burnetii* WC vaccine is readily phagocytized by macrophages and that this results in non-specific activation of these cells. Preliminary experiments with prostaglandin synthesis inhibitors, macrophage toxins, cell-depletion techniques, and co-culture protocols suggest that the suppression and negative regulation of mouse spleen cells by phase I WC vaccine may be mediated by T-suppressor cells.

Recently we were successful in identifying splenic cell fractions which participate in the suppression of lymphocyte proliferation after injection of WC vaccine. Splenic lymphocytes from WC vaccine-injected mice were co-cultured with normal lymphocytes so that the regulatory influence of suppressed lymphocytes on the normal lymphocytes could be measured in vitro. T-cells (both helper, T-H; and suppressor, T-S) cultured with normal lymphocytes were effective in suppressing in vitro proliferation of the normal cells to mitogens and antigens. The elimination of theta-bearing cells (T-cells) to produce a B-cell fraction did not completely inactivate the suppressor. In addition, nylon wool adherent and non-adherent cells effectively suppressed the in vitro response of normal lymphocytes. We also have detected a soluble suppressor substance produced by lymphocytes from WC vaccine-injected mice, which inhibits the proliferation of normal lymphocytes. Therefore, the induction mechanisms appear to be antigen-specific, but the suppressor mechanism is non-specific and several cell types may be involved.

Genetics of Immunopathological Responses Previous studies have shown that i.p. administration of phase I WC vaccine to C57BL/10 ScN mice results in gross pathologic changes. We have subsequently shown that injection of phase I WC vaccine caused a marked and persistent suppression of the in vitro lymphocyte proliferation response of host spleen cells to both specific and non-specific stimuli. The concurrent pathogenic and suppressive events suggested that the hepato-splenomegaly and immunological unresponsiveness were linked. However, our current studies of the immunologic status of BALB/c sublines and congenic strains after either phase I *C. burnetii* infection or vaccination with phase I WC vaccine have convinced us that gross pathologic responses can be genetically unlinked from the immunologic unresponsiveness. In the present study, we found that the BALB/c sublines and congenic strains were intermediately sensitive to *C. burnetii* infection; spleens of infected animals were significantly enlarged, whereas those of vaccinated animals only were slightly increased. The splenic lymphocytes from phase I WC vaccine-injected mice were hyporesponsive to PWM, PHA, LPS-Ec, and recall antigens. The observation that splenic lymphocytes were suppressed in the absence of splenomegaly suggests that the BALB/c mice and congenic strains are able to distinguish between *C. burnetii* virulence factors which induce splenomegaly.

These results indicate that BALB/c sublines and congenic strains injected with phase I WC vaccine differ from C3H/HeN, A/J, and C57BL/10 ScN mice in several pathologic and functional activities of splenic lymphocytes: (i) splenic lymphocytes from phase I WC vaccine-injected mice were not negatively regulated by recall antigen and the response to ConA only was either depressed (BALB/cPt), unchanged (BALB/cJ), or enhanced (CB-20, CAL-20); (ii) splenic lymphocytes from phase I WC vaccine-injected mice, in contrast to saline-injected mice, required a tenfold greater concentration of PHA for optimal stimulation. A similar shift in PHA responsiveness of splenic lymphocytes was not observed for the C3H/HeN, A/J, or C57BL/10 ScN mice. While the ability of splenic lymphocytes from saline-injected mice to respond to ConA and PHA was preserved within the BALB/c sublines, the ability of CAL-20 splenic lymphocytes to respond to LPS-Ec was significantly less than CB-20 and the BALB/c sublines. Results of other studies show that the Igh genotype influences the immuno-regulatory role of B- and T-cells and their products. In our studies, the generation of in vitro lymphocyte proliferative responses to mitogens and specific antigens either before or after phase I WC vaccine injection also seem to be influenced by Igh genotype. Although we were able to discern patterns of lymphocyte responsiveness which were related to the Igh genotype, a definitive result was not obtained.

Some investigators have used increased spleen indices as a measure of sensitivity of mice after *Mycobacterium lepraemurium* infection. Studies conducted by others showed that Ity^s mice (C57BL and BALB/c) responded with splenomegaly, whereas Ity^r mice (DBA/2, CBA and C3H/HeN) showed no increases in spleen weight. In our studies, we were searching for an inbred mouse that does not develop splenomegaly, but one in which lymphocyte responses are suppressed after injection of phase I WC vaccine. The BALB/c sublines and congenic mice respond to *C. burnetii* injection with hepato-splenomegaly and suppression, whereas phase I vaccine injection produced suppression without hepato-splenomegaly. Thus, increased spleen indices after infection or vaccination were not an adequate measure of pathologic responses of the BALB/c sublines and congenic strains. These responses were not predictive correlates of immunologic unresponsiveness or sensitivity to infection. These observations indicate that the Igh-controlled functional specificities tested in this study may govern, at least in part, the in vivo development of splenomegaly. The role of B- and T-cell specific responses of Igh alleles in other congenic strains should be tested since the incidence of mortality of A/J ($H\text{-}2^d$ $Igh\text{-}1^e$) mice after infection correlated with low interferon production and the $Igh\text{-}1^e$ allele. We plan to test the BALB/c sublines and congenic strains which produce high levels of interferon and carry the $Igh\text{-}1^e$ allele.

The humoral and cellular arms of the immune response contribute either directly or indirectly to the pathogenesis of a variety of intracellular parasites. Although definitive studies have not been performed to differentiate the interaction of gene products with the virulence factors of pathogens at the molecular level, several studies have demonstrated that inbred mice respond to various parasites through apparently unpredictable mechanisms. For example, natural resistance of inbred mice to infections with *Salmonella typhimurium*, *Leishmania donovani*, *Mycobacterium bovis*, and *M. lepraemurium* are closely linked to a group of genes on mouse chromosome 1. Resistance of mice to infection by *Rickettsia tsutsugamushi*, by contrast, correlates with a Ric gene on chromosome 5. In other diseases caused by *Listeria monocytogenes*, *Rickettsia akari*, and *C. burnetii* correlation of susceptibility with particular gene loci has not been adequately studied for us to arrive at a firm conclusion.

Immunomodulation in Humans Human infection with the rickettsia *C. burnetii* presents as an acute, flu-like, primary Q fever; as a subacute granulomatous hepatitis; or as a chronic endocarditis with hepatitis. To investigate whether persistent infection is associated with a possible immunologic defect, *Coxiella*-specific in vitro lymphocyte proliferation was tested in peripheral blood mononuclear cells (PBMC) from four North American patients with endocarditis, four patients with granulomatous hepatitis, eight patients convalescent from acute primary Q fever, and ten seronegative controls. Despite having the highest serum phase I complement fixation titers, all four endocarditis patients had profound lymphocyte unresponsiveness to *Coxiella* antigens, yet displayed normal proliferation to control antigens. Hepatitis and primary Q fever were associated with vigorous responses in vitro to *Coxiella* antigens. Suppression of lymphocyte unresponsiveness was in part mediated by an antigen-nonspecific, glass-adherent cell. The failure of T-cells and $T8^+$(suppressor)-depleted cells to respond to *Coxiella* antigens suggests additional mechanisms of suppression are operative. We hypothesize that specific T-cell unresponsiveness is an important factor in persistent infection with *C. burnetii*, and we offer in vitro lymphocyte stimulation as a more specific diagnostic test to distinguish cases of endocarditis among those with chronic Q fever hepatitis.

In another study, the adherent suppressor cells were shown to work via prostaglandin E_2 (PG_{E2}) production. Addition of the cyclooxygenase inhibitor, indomethacin, to cultures of PBMC from patients with endocarditis or continuous laboratory exposure resulted in consistent increases in *Coxiella*-specific lymphocyte proliferation. The degree of increase in proliferation induced by indomethacin correlated strongly with the amount of PGE_2 produced in a 4-h culture stimulated by *Coxiella* antigen, but it also correlated with the sensitivity to inhibition of mitogenesis by PGE_2. The suppressor mechanism was antigen-nonspecific, since induction of suppression in vitro by *Coxiella* antigen also suppressed *Candida*-induced proliferation when both antigens were present in the same culture. Addition of indomethacin to these antigen co-cultures totally reversed the *Coxiella*-induced suppression, confirming that the nonspecific effector mechanism of suppression was prostaglandin - mediated. Elicitation of suppression, however, was antigen-specific, and involved a T-cell-monocyte suppressor circuit. Supernatants from *Coxiella*-stimulated immune T-cells and from the suppressor subset ($OKT8^+$-enriched) of those T-cells, but not unstimulated immune cells, induced augmented PGE_2 production by unrelated nonimmune PBMC. We concluded that the lymphocyte unresponsiveness characterizing patients with Q fever endocarditis is modulated in part by an antigen-specific T suppressor cell which secretes a lymphokine to stimulate PGE_2 production by adherent cells.

Characterization of lipopolysaccaride from several strains of *Coxiella burnetii* Ultrastructures of LPSI and LPSII positively stained with uranyl formate or uranyl acetate were ribbon-like. When negatively stained with uranyl acetate, LPSI was ribbon-like, while LPSII exhibited hexagonal lattice structures. However, LPSII stained negatively with sodium phosphotungstate and ammonium molybdate exhibited hexagonal lattice structures that were not identical to those observed when LPSII was negatively stained with uranyl acetate. The hexagonal lattice structures formed in vitro were due to the interactions of LPSII and the staining reagents rather than to protein-LPS interactions. The differences in the ultrastructures of LPSI and LPSII are undoubtedly based on variations in their chemical compositions.

Phase I LPS from 9 Mile, Ohio, Henzerling, P, K, and Australian strains of *C. burnetii* contained mannose, mannoheptose, unknown neutral sugars, glucosamine, galactosaminuronic acid, compound X (galactosaminuronyl-glucosamine), KDO, and phosphate. Phase II LPS from 9 Mile II and M44 strains contained mannose, mannoheptose, glucosamine, KDO, and phosphate. The amounts of KDO and glucosamine of *C. burnetii* LPS were lower than those found in *Salmonella* LPS. Also, *C. burnetii* LPS contained low amounts of fatty acid. The fact that *C . burnetii* LPS contained low or undetectable amounts of fatty acids completely differentiates this non-toxic LPS from gram-negative bacterial LPS. Therefore, we speculate that *C. burnetii* LPS is a new type of LPS.

The LPS of virulent phase I *C. burnetii* contained a disaccharide which was resistant to acid hydrolysis. Disaccharide, purified by ion-exchange column chromatography, contained amino, carboxyl, and reducing groups. After N-acetylation, reduction of carboxyl and reducing groups, and acid hydrolysis, the compound exhibited two components (galactosamine and glucosaminitol). After N-acetylation and reduction of carboxyl groups and reducing groups, this compound co-migrated with N, N-di-acetychitobiose on a paper chromatogram, was hydrolysed with α-N-acetyl-galactosaminidase, but was resistant to β-N-acetylhexosaminidase. Smith degradation of this N-acetylated compound X showed that the linkage between the two sugars was 1 to 6. Based on the data, the compound X was determined to be galactosaminuronyl α(1→ 6) glucosamine.

The LPS of 9 Mile I and Ohio strains consisted of seven components (between 10- and 20K daltons, and high molecular weight broad bands. LPS of the Henzerling strain contained three bands in addition to the bands of 9 Mile I LPS. The major band from each of three strains was 14K. LPS of the P strain showed seven bands (between 10K and 20K daltons). The electrophoretic pattern for the P strain was markedly different from those of the other strains, and the major band of this LPS was 13K daltons. LPS of 9 Mile II and M44 was indicative of the rough (phase II) profile and contained only one band at about 3K daltons. LPS of the K strain showed a mixture of two LPS patterns which resembled the P and 9 Mile II. LPS of Australian strain was composed of three bands (3-, 12-, and 20K daltons). On the basis of SDS-PAGE patterns, the 9 Mile II and the M44 LPS showed single low molecular weight bands. The 9 Mile I, Ohio, and Henzerling strains contained no LPS II. However, the K and Australian strains were mixtures of LPSI and LPSII. The P strain contained a small amount of LPSII in addition to LPSI.

Identification of *C. burnetii* antigens We have been studying a 29.5K-dalton, protein-containing fraction that we determined to be antigenically significant. A specific immune response was induced with the fraction in both mice and rabbits. Spleens from the mice were used for fusions with myeloma cells; hybrids were screened by ELISA techniques for production of antibodies reactive with the 29.5K fraction. By using unconcentrated tissue culture supernatants, cloning by limiting dilution has yielded 24 cell lines that secrete enough immunoglobulin to be found reactive with the 29.5K protein. Using whole-cell phase I and phase II antigens, as well as the 29.5K protein, to study the reactivity of these antibodies, we observed the following patterns: (i) positive reactions with all three antigens, (ii) positive only with 29.5K, and (iii) positive with 29.5K and phase II antigens but negative with phase I. For class (i), the general pattern was high reactivity toward 29.5K and phase II antigens and weak reactivity toward phase I antigen. Determination of antibody heavy chain subclass has been done; antibodies of subclass IgG1, IgG2a, and IgM were obtained. This preliminary characterization has allowed us to select eight cell lines for growth in ascites fluids and eventual ultimate characterization of reactivity by techniques such as radioimmune precipitation and immune blotting. The 29.5K fraction was subjected to the additional purification step of preparative polyacrylamide gel electrophoresis, and a Coomassie blue-stained band was injected into rabbits. The rabbit serum reactivity has not yet been characterized.

It is important to know whether the nucleic acid heterogeneity, manifested by restriction enzyme fragment-length differences, among various isolates of *C. burnetii* is manifested in heterogeneity among the proteins. The proteins of *C. burnetii* were extracted with sodium dodecyl sulfate (SDS), the charge effect of SDS was counteracted by a zwitterionic detergent, and the proteins were separated with isoelectric focusing on the basis of charge. The proteins were further separated on the basis of molecular weight, providing a two-dimensional separation. Staining with ammoniacal silver yielded a pattern of approximately 200 spots that was reproducible enough to permit comparison between strains. The result was one of great similarity between the patterns of different strains. A number of differences, however, were observed: the 9MIC7, 9MIIC4, K, and P strains were identical by visual inspection, and contained two additional proteins when compared to the Henzerling and M44 strains. More sensitive methods of analysis undoubtedly will show additional heterogeneity among the various strains.

In another approach to examine possible strain differences, an attempt was made to identify DNA-binding proteins. Proteins, separated by electrophoresis in polyacrylamide gels and then transferred to nitrocellulose, were incubated with [^{32}P]DNA labelled by nick translation, and then washed extensively to remove unbound DNA. Examination by autoradiography showed the DNA had bound to proteins of molecular weight 20K, 17K, 16K, and 15K from the 9MIC7 and 9MIIC4 strains. The M44 and Henzerling patterns were different in that they both exhibited more binding to the 20K protein. The P strain was different in that only binding to the 17K protein was observed. The method applied was a general one to detect DNA binding proteins; future work can use techniques that permit the identification of proteins that exhibit specificity of binding to defined sequences of nucleotides.

PRESENTATIONS:

1. **Snyder, C. E., Jr., and J. C. Williams.** Isolation and characterization of proteinaceous antigens of *Coxiella burnetii*. Presented, Annual Meeting of the American Society of Tropical Medicine and Hygiene, Baltimore, MD, Dec 84.

2. **Amano, K., K. Fukushi, and J. C. Williams.** Chemical ultrastructural properties of lipopolysaccharides from phase I and phase II *Coxiella burnetii*. Presented, American Society of Rickettsiology, Laguna Beach, CA, Feb 85.

3. **Snyder, C. E., Jr., and J. C. Williams.** Isolation of envelope proteins from *Coxiella burnetii:* Use of detergent solubilization and ion-exchange chromatography. Presented, American Society of Rickettsiology, Laguna Beach, CA, Feb 85.

4. **Waag, D., and J. C. Williams.** The biochemical characterization of an immunosuppressive component associated with *Coxiella burnetii* phase I. Presented, American Society of Rickettsiology, Laguna Beach, CA, Feb 85.

5. **Amano, K., K. Fukushi, and J. C. Williams.** Chemical ultrastructural properties of polysaccharides from phase I and phase II *Coxiella burnetii.* Presented, 85th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, Mar 85.

6. **Snyder, C. E., Jr., and J. C. Williams.** Isolation of envelope proteins from *Coxiella burnetii:* use of detergent solubilization and ion-exchange chromatography. Presented, 85th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, Mar 85.

7. **Waag, D., and J. C. Williams.** Cellular immune response of C57BL/10 ScN mice after injection with phase I *Coxiella burnetii.* Presented, 85th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, Mar 85.

PUBLICATIONS:

1. **Amano, K.-I., K. Fukushi, and J. C., Williams.** 1985. Electron microscopic studies of lipopolysaccharides from phase I and phase II *Coxiella burnetii. J. Gen. Microbiol.* (Submitted).

2. **Amano, K.-I., J. C. Williams, T. F. McCaul, and M. G. Peacock.** 1984. Biochemical and immunological properties of *Coxiella burnetii* cell wall and peptidoglycan-protein complex fractions. *J. Bacteriol.* 160:982-988.

3. **Amano, K.-I., and J. C. Williams.** 1984. Sensitivity of *Coxiella burnetii* peptidoglycan to lysozyme hydrolysis and correlation of sacculus rigidity with peptidoglycan-associated proteins. *J. Bacteriol.* 160:989-993.

4. **Amano, K.-I., and J. C. Williams.** 1984. Chemical and immunological characterization of lipopolysaccharides from phase I and phase II *Coxiella burnetii. J. Bacteriol.* 160:994-1002.

5. **Damrow, T. A., J. C. Williams, and D. M. Waag.** 1985. Suppression of *in vitro* lymphocyte proliferation in C57BL/10 ScN vaccinated with phase I *Coxiella burnetii. Infect. Immun.* 47:149-156.

6. **Koster, F. T., J. C. Williams, and J. S. Goodwin.** 1985. Cellular immunity in Q fever: modulation of responsiveness by a suppressor T cell-monocyte circuit. *J. Immunol.* (In Press).

7. **Koster, F. T., J. C. Williams, and J. S. Goodwin.** 1985. Cellular immunity in Q fever: specific lymphocyte unresponsiveness in Q fever endocarditis. *J. Infect. Dis.* (In Press).

8. **Williams, J. C., T. A. Damrow, and D. M. Waag.** 1985. Characterization of a phase I *Coxiella burnetii* chloroform-methanol residue vaccine that induces active immunity against Q fever in C57BL/10 ScN mice. *Infect. Immun.* (Submitted).

9. **Williams, J. C., V. Sanchez, G. H. Scott, E. H. Stephenson, and P. H. Gibbs.** 1985. Variation in responsiveness of Balb/c sublines and congenic mice to phase I *Coxiella burnetii* infection and vaccination. *In* M. Potter (ed), Immunological response of the Balb/c mouse. Springer-Verlag, New York (In Press)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 302662	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYM DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763D	3M263763D807	AC	013
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Exploratory Development of Vaccines Against Rift Valley Fever

12. SUBJECT AREAS
0613 Microbiology; 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousan
b. CONTRACT/GRANT NUMBER		85	1.0	388
c. TYPE	d. AMOUNT	86	5.0	1141
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Disease Assessment Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Peters, C J
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7244
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Balady, M
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Junin Virus; (U) Rift Valley Fever Virus; (U) Vaccines; (U) Lab Animals; (U) Monkeys; (U) RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop and produce live, attenuated viral vaccines to be administered to at-ris military personnel.

24. (U) Viruses will be propagated in certified cell cultures, cloned, and selected for markers of attenuation. Vaccine will be safety-tested and efficacy will be estimated in animal models.

25. (U) 8410 - 8509 - Portions of the coding sequences for Rift Valley fever virus (RVFV) glycoprotein genes have been inserted into vaccinia virus. This was used to infect cultured cells. Subsequently, RVFV antigens were found on the cell surface, a phenomenon not observed with parental RVFV virus. Vaccinia hybrid viruses also induced RVFV-specific, cytotoxic T cells in infected mice. In order to examine the variability and significance of different RVFV epitopes, variants were selected by using four neutralizing antibodies specific for the G2 protein. Resistant mutants were found that had widely varying patterns of stability, virulence, and antigenicity. RVFV M segment RNA has been molecularly cloned. This and related DNA clones have been inserted into vaccinia to produce RVFV antigen expression, neutralizing antibody production, and protection of mice from virulent RVFV challenge.

Work continued from DA302666.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AC-013: Exploratory Development of Vaccines Against Rift Valley Fever

PRINCIPAL INVESTIGATOR: C. J. Peters, COL. M.D.

ASSOCIATE INVESTIGATOR: M. Balady, CPT, Ph.D.

Background

Rift Valley fever virus (RVFV) is an important military pathogen in several respects. It is widely distributed throughout the African continent south of the Sahara and can naturally infect troops via the bite of infected mosquito vectors. In 1977, this virus spread to Egypt for the first time and caused a massive epizootic/epidemic, demonstrating its capacity to spread into virgin territory and thus its potential for strategic use as a BW agent. Finally, it causes aerosol infections in those persons dealing with infected animal carcasses or laboratory specimens, confirming formal aerosol studies that suggest we should be concerned that hostile nations might weaponize RVFV as a BW agent.

There is an existing formalin-inactivated vaccine produced by a technology resembling that used for the Salk polio vaccine. This prototype immunogen has served well in protection of at-risk laboratory workers, but is impractical for large scale use. The lots vary in potency; three injections are needed to immunize, and production techniques are cumbersome. We have chosen this vaccine as a benchmark of conventional technology and are systematically attempting to improve this product. Two approaches are currently under study. In one, virus is intensively mutagenized to decrease its pathogenetic potential and therefore create an attenuated vaccine. An attempt will also be made to map some critical genomic sites by selection of variants by using neutralizing monoclonal antibodies. The second approach will utilize small peptides to mimic critical antigenic determinants on RVFV and provide synthetic vaccines. This approach obviously depends on cloning, sequencing, and monoclonal antibody analysis.

Summary:

Portions of the coding sequences for Rift Valley fever virus (RVFV) glycoprotein genes have been inserted into vaccinia virus. This was used to infect cultured cells. Subsequently, RVFV antigens were found on the cell surface, a phenomenon not observed with parental RVFV virus. Vaccinia hybrid viruses also induced RVFV-specific, cytotoxic T cells in infected mice. In order to examine the variability and significance of different RVFV epitopes, variants were selected by using four neutralizing antibodies specific for the G2 protein. Resistant mutants were found that had widely varying patterns of stability, virulence, and antigenicity. RVFV M segment RNA has been molecularly cloned. This and related DNA clones have been inserted into vaccinia to produce RVFV antigen expression, neutralizing antibody production, and protection of mice from virulent RVFV challenge.

Progress:

Vaccine development in the next few years may change remarkably, but the exact form these changes will take is not clear. Studies described here are directed toward understanding the principles underlying success or failure of such technologically advanced approaches utilizing vaccinia as a gene vector and monoclonal antibodies to probe antigenic structure of the pertinent genes. In both series of studies, RVFV has been used as the test organism.

Portions of the coding sequences of RVFV glycoprotein genes have been inserted into vaccinia virus and this construct used to infect cultured cells. RVFV antigens were detected on the cell surface, a phenomenon not observed with parental RVFV virus. Vaccinia-RVFV hybrid viruses also induced RVFV-specific, cytotoxic T cells in infected mice. In order to examine the variability and significance of different RVF epitopes, variants were selected by using four neutralizing monoclonal antibodies specific for the G2 protein. Resistant mutants were found at a frequency of 10^{-4} to 10^{-6} and had widely varying patterns of stability, virulence, and antigenicity.

RVFV M segment RNA has been molecularly cloned and used in several previously reported studies. This and related DNA clones have been inserted into the vaccinia virus genome with the appropriate molecular machinery to produce RVFV antigen expression, neutralizing antibody production, and protection of mice from virulent RVFV challenge. These studies analyze the nature of antigen expression and the quality of the immune response following infection with these vaccinia viruses bearing RVFV genes. The ZH501 RVFV strain, the gene donor for molecular cloning, was used as the prototype virulent virus. For convenience, parental vaccinia virus is referred to as VAX I and constructs with varying lengths of RVFV in segment RNA are designated VAX-5 (Table 1). An unusual RVFV isolate attenuated for adult mice (Tunyo virus) was also studied.

RVFV and other Phleboviruses have never convincingly been shown to induce cell-surface antigens. Vaccinia-bearing RVFV genes infected several cell types and resulted in expression of such antigens as detected by fluorescence (Table 1.) The presence of these antigens as integral membrane proteins was confirmed by demonstrating their ability to mediate lysis in the presence of complement (c) (Table 2). Surprisingly, Tunyo virus infection led to significant cell-surface antigen expression, as well. These results are of both theoretical and practical importance in other systems. Cell-surface antigen expression is of critical importance in triggering T cell subsets mediating delayed type hypersensitivity and cytotoxicity. From a "nuts-and-bolts" perspective, lack of cells bearing virus-specific antigens has precluded a number of experimental studies to delineate the role of these and other mechanisms in the immunobiology of Rift Valley fever. Finding RVFV glycoproteins in cell membranes will lead to a broader evaluation of the factors regulating this phenomenon and a search for its occurrence in other cell-virus combinations.

Initial steps to demonstrate RVFV-specific cytotoxic lymphocytes in (CTL) infected mice have been successful. Highly active, H2-restricted CTL have been detected, particularly in draining lymph nodes from foot-pad inoculated animals. Tunyo and VAX2-5 have induced CTL (Table 3). By comparison, mice infected with unaltered vaccinia or injected with inactivated RVFV vaccine do not demonstrate significant CTL activity. Clearly, additional studies are required to define the phenomenon in more detail, but these results suggest that previous concepts of phlebovirus glycoprotein metabolism must be extended, that vaccinia-RVFV hybrids can induce expression of important antigens on the surface of infected cells, and that CTL responses do occur in mice infected with such viruses as well as selected RVFV strains.

It seems likely that tomorrow's vaccines will be produced from selected pieces of the genomes of viral pathogens, by using vaccinia and other vectors multiplying in the host or introducing non-replicating immunogens. In any case, it will be necessary to understand the role of individual antigenic epitopes in pathogenesis, including their protective capacity, their variation, and the significance of their variation in the biology of the virus. To approach these questions, we used monoclonal antibodies (MAB) to select variants of RVFV and studied the properties of these rural variants. Four MAB produced against the Entebbe strain and reacting with the G2 glyco-protein were employed. The frequency of non-neutralizable variants present in ZH501, Entebbe, or Smithburn strain virus pools was 10^{-4} to 10^{-6}. About 1/3 of the 68 variants studied were stable when passaged in the absence of selecting antibody. Several variants with diminished lethality for adult mice were obtained from virulent parents such as ZH501 or Entebbe, but virulent strains were not noted among the variants from the avirulent Smithburn virus pool. These studies require extension to larger numbers of derived viruses.

The majority of the variant viruses studied were derived from a cloned ZH501 pool. When they were examined in detail with additional monoclonal and polyclonal antisera, several variants were neutralized less readily. Comparison within the group suggested that there were three distinct epitopes interacting in this system.

The analysis of interactions between highly specific MAB and RVFV clearly should be extended to the T-cell level, but data summarized in this annual report have established the necessity for understanding more about the fine structure of viral antigens, their interaction with the immune system, and the significance of selective epitopic alteration on flu virus.

PRESENTATIONS: None

PUBLICATIONS: None

Table 1. Surface fluorescence

Virus Strain	% of Cells Positive for RVF Antigen Cell Type		
	L929	3T3	P815
LUNYO	32%	36%	2%
ZH-501	5%	0%	4%
VAX 1 (WT)	0%	0%	0%
VAX 2	4%	2%	1%
VAX 3	7%	4%	2%
VAX 4	2-3%	11%	1%
VAX 5	85%	28%	1%

Cells were infected with virus at MOI=1.00 for 16 h, fixed with 0.05% glutaraldehyde, and processed for antigen by counting 300 cells per sample and calculating the % positive. VAX 1 = wild type vaccinia, New York Health Dept strain; VAX 2, 3, and 5 represent vaccinia viruses with inserts of the M segment of varying lengths of RVFV: and the VAX 4 represents a vaccinia containing the gene for the G2 glycoprotein of RVFV.

Table 2. Complement-mediated lysis[a]

Virus	% Specific lysis[b]
LUNYO	29%
VAX 1	0%
VAX 2	13%
VAX 3	9%
VAX 4	8%
VAX 5	28%

[a] L929 cells infected for 18 h, MOI 5.00.

[b] C' 1:40 dilution; antibody at 1:10 dilution.

Table 3. CTL assay spleen cell assay[a]

	% Specific lysis of L929 infected cells ^{51}Cr-labeled				
	DAYS POST INOCULATION OF VIRUS				
VIRUS	1	4	5	7	9
LUNYO	5%	11%	14%	7%	3%
VAX 1	1%	3%	0%	0%	0%
VAX 2	7%	13%	11%	0%	0%
VAX 3	8%	7%	13%	7%	0%
VAX 4	6%	12%	11%	6%	5%
VAX 5	14%	13%	23%	6%	[illegible]
INACTIVATED VACCINE	0%	0%	4%	0%	0%

[a]Spontaneous release was 20% for these experiments; E:T ratio was 50:1. Spleen cells were treated to remove RBC prior to 4 h CTL assay on 16-h infected targets.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 302664	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763A	3M263763D807	AD	014
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Development of Antiviral Drugs

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	3.2	637
86		

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Canonico, P G
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2290
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Kende, M
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Ussery, M A

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) Antiviral Drugs; (U) Pharmacology; (U) Viral Diseases; (U) Lab Animals; (U) Mice; (U) RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Identify effective drugs against viruses that are potential threats to military personnel; obtain data on toxicology, pharmacology, and metabolism of antiviral drugs and conduct preclinical and clinical studies to assess safety and efficacy in compliance with FDA regulations. Conduct laboratory studies to develop novel applications of drug delivery systems for antiviral chemotherapy. Develop adjuvants for use with subunit viral vaccines.

24. (U) Assess efficacy of potential antivirals against viruses in tissue cultures and in rodent models for Rift Valley fever virus (RVFV) and VEE. Evaluate toxicity and pharmacology of promising compounds in preclinical protocols conducted in rodents and nonhuman primates. Provide technical support for clinical protocols. Evaluate state-of-the-art technologies for improved drug delivery, such as liposomes, topical application, implantable infusion pumps, and molecular carriers. Perform animal studies to assess efficacy of immunopotentiating compounds and drugs combinations as potential antivirals or vaccine adjuvants.

25. (U) 8410-8509-Mice were used to determine the optimal treatment regimens with the immunomodulator, poly(ICLC). For prophylactic treatment, three 1 μg doses over 12 days were found to be effective against RVFV infection. For therapeutic application, three 20 μg doses were required. Combination therapy with ribavirin and poly (ICLC) was highly efficacious. Protection was afforded with very low nontoxic doses of these drugs when administered as late as 48 h after infection. Picabanil, a new immunodulatory compound, was moderately efficacious against RVFV in mice. The compound elicited only low levels of interferon, but induced a strong cytotoxic activity of NK cells. An African green monkey model was developed for yellow fever. The model offers a valuable tool to evaluate antiviral compounds and immunomodulators, because of the moderate nature of the infection.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AD-014: Exploratory Development of Antiviral Drugs

PRINCIPAL INVESTIGATOR: Dr. Peter G. Canonico

ASSOCIATE INVESTIGATORS: Dr. John Huggins
Dr. Meir Kende
CPT Michael Ussery

Background:

Drug screening efforts at USAMRIID have identified a number of compounds with broad-spectrum antiviral activity against "exotic" RNA viruses. Among these, the nucleoside analogue, ribavirin, has been found to be especially active against a number of debilitating and lethal viruses (1). Ribavirin has also been evaluated most extensively in experimental animals and man. It has been found to be effective against Rift Valley fever, Lassa fever, and the hemorrhagic phase of Machupo virus infections of monkeys. The research program addresses the continued evaluation of ribavirin and its application as a broad-spectrum antiviral agent. For example, studies have beeu conducted to evaluate ribavirin therapy against Junin virus infection in non-human primate.

Work on further development of immunomodulators has continued. The interferon inducer, poly(ICLC), has been evaluated further to determine the optimal prophylactic and therapeutic schedule which could be used for human use. Similarly, the availability of recombinant interferon alpha A and gamma has, for the first time, made the use of interferon in the treatment of viral diseases a practical possibility. Hence, studies normally required prior to use of drugs in man were initiated. The first series of experiments evaluated the pharmacokinetic parameters in monkeys for human recombinant alpha and gamma interferons, as well as serum chemistry levels, after use of the interferons given alone or in combination. These parameters must be established to determine the optimal dosage levels for the treatment of infectious agents of interest in monkeys and before any Investigational New Drug (IND) permit could be granted for the use, on an experimental basis, of alpha and gamma combinations in human patients.

Summary:

Mice were used to determine the optimal treatment regimes with the immonomodulator poly(ICLC). For prophylactic treatment, three 1-g doses over 12 days were found to be effective against Rift Valley fever virus infection. For therapeutic application, three 20-g doses were required. Combination therapy with ribavirin and poly(ICLC) was highly efficacious. Protection was afforded with very low, nontoxic doses of these drugs when administered as late as 48 h after infection. Pharmacokinetic studies with human recombinant interferons in uninfected African green monkeys with interferon (IFN) alpha alone, IFN gamma, and both-combined showed that serum levels that may be effective could be achieved without measurable side effects such as pyrexia, anorexia, or serum chemistry changes.

Ribavirin Therapy of Junin Infection in Primates. Argentine hemorrhagic fever (AHF) is a debilitating and often fatal human disease caused by Junin virus. In man, a spectrum of clinical illness is seen that ranges from a mild, flu-like affliction to fulminant disease and death in 15 to 30% of untreated individuals (2). Following a 7- to 14-day incubation period, disease is characterized by fever, headache, loss of appetite, muscle aches, back and abdominal pain, dizziness, constipation, and diarrhea; accompanied by flushing of the face and chest, redness of the eyes, and congestion with bleeding of the gums (3). Leukopenia and thrombocytopenia are important hematologic accompaniments of disease, and alpha-interferon levels typically are very high (6). Improvement will begin in most patients during the second week, while those with more severe disease will evolve a predominantly hemorrhagic, neurologic, or mixed hemorrhagic/neurologic with shock syndrome which frequently leads to death (3). In most years, 200 to 400 cases are observed within the endemic region. However, for reasons which remain obscure, periodic large epidemics occur, involving thousands of individuals in this region.

While mortality in untreated AHF is high, successful therapy for the disease recently has been achieved through use of human AHF immune plasma. Although effective in preventing death during the acute stages of illness, the utility of immune plasma therapy is limited to patients whose disease has progressed no further than 8 days following symptom onset.

As a result of these studies, individuals whose disease is > 8 days in duration are currently treated only supportively, and suffer a risk of death equal to that of patients with shorter duration disease and who are not receiving specific immunotherapy.

Ribavirin has been efficacious against Junin virus in the guinea pig model (1), and previous studies with the closely related virus, Machupo, in primates have demonstrated prevention of the hemorrhagic component. Discussions of potential ribavirin therapy for the human disease led to the realization that considerable gaps existed in our knowledge of ribavirin therapy. A primate, ribavirin, efficacy study was undertaken by using the newly developed Junin model with rhesus macaques that closely mimic the AHF clinical syndrome seen in man. Prophylactic as well as therapeutic studies of ribavirin in Junin virus infection of rhesus macaques were undertaken. Sham-treated control animals infected with about 10^4 PFU of the highly lethal Espindola strain of Junin virus showed 100% mortality with a classical hemorrhagic diathesis during the third and fourth weeks. AHF infection caused severe weight loss (25%). Treatment was given, prophylactically or therapeutically, after the onset of clinical signs, on day six postinfection. The study involved characterization of the disease course under P-4 conditions.

Rhesus monkeys were administered ribavirin on a prophylactic schedule (60 $mgkg^{-1}day^{-1}$ for 4 days, 30 $mgkg^{-1}day^{-1}$ for 3.5 days, then 16 $mgkg^{-1}day^{-1}$ for 11 days) seroconverted, but failed to develop viremia or clinical signs of illness. In animals receiving ribavirin therapeutically (60 $mgkg^{-1}day^{-1}$ for 1.5 days, then 15 $mgkg^{-1}day^{-1}$ for 14 days), viremia was detected up until the time of drug administration, then disappeared for the duration of observations. Clinical signs resolved over a two-week period, and animals showed only a 15% weight loss. As occurred in Machupo-treated animals, however, a late onset neurological syndrome appeared in all animals, and was fatal in two of three with the third recovering fully. Thus, ribavirin was successful in treating systemic, but not central nervous system components of the disease, when therapy was initiated after onset of clinical signs; prophylactic treatment prevented clinical disease completely.

Discussions with Dr. Julio Maiztegui of the Instituto Nacional de Estudios Sobre Virosis Hemorragicas, Pergamino, Argentina, the national treatment center for Argentine hemorrhagic fever, led to the initiation of a ribavirin clinical trial based on the evidence accumulated during these and other studies.

Antiviral Activity of immunomodulators. A daily dose of 20 μg/mouse of poly(ICLC) for 10 to 12 days was used in early prophylactic and therapeutic efficacy studies. Although this dose is not toxic to mice regarding weight loss, loss of vigor, and death, it is known to cause toxic complications in humans. Hence, an efficacious schedule which would not cause toxicity to man must be determined. A study to find the most optimal prophylactic and/or therapeutic schedule for a single or double dosage regimen of poly(ICLC) which would give maximum protection against a virus challenge would require 36 experimental groups and many more if a three-injection regimen was needed.

One approach to selecting the best treatment schedule is ranking of treatment efficacy by using the Cox model (4), which expresses the efficacy in the form of relative risk of death in comparison to a standard day-0 treatment. This approach assumes the existence of a linear response between the timing interval of a single drug dose with respect to challenge. With the Cox model approach, an optimum treatment schedule can be determined with as few as 18 experimental groups, receiving either one or two doses, and as few as 24 groups, if a number of preselected triple-dosage groups are included.

The relative risk of death is calculated with the equation:

$$H(T,Z) = \lambda(T) \exp(\beta Z) \quad \text{where}$$

T = time
Z = indicator variable for "regimen"
H(T,Z) = hazard (instantaneous rate of death) at time "T" for an animal in regimen "Z"
λT = underlying hazard function for standard regimen (Z=0)
β = regression coefficient for regimen "Z"

The factor exponent (βZ) is the incremental relative risk of death (relative to the standard defined to Z=0 [in our case, treatment and challenge at day 0]). A test of β=0 tests whether the incremental relative risk is significantly different from day 0 standard treatment.

The ranking creates three categories. These are treatment schedules with efficacies which are inferior, equal, or better than the standard (day 0) treatment. Specifically, the respective relative risk of death associated with these categories is either significantly higher ($p<0.05$), indistiguishable, or significantly lower ($p<0.05$) compared to day-0 treatment. By this approach, the treatment schedule of choice was found to be a three-injection regimen given on days -4, 1, and 6. This schedule was selected because it provides the longest interval between injections while retaining maximum efficacy (90%). The regimen whereby poly(ICLC) is given on days -1 and +1 was not selected, although it gives greater efficacy because the proximity of the two doses enhances the probability that toxicity could be manifested in man.

<u>Pharmacokinetics following simultaneous single dose administration of recombinant interferon alpha A and gamma to African green monkeys</u>. Three African green monkeys received one dose of 0.1 mg/kg gamma interferon and one week later by 10^6 µg/kg alpha interferon, followed one month later by five daily doses of 0.1 mg/kg gamma plus 10^6 µg/kg gamma. Serum samples were taken 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after each single interferon dose and on day 1 and 5 of the combination treatment. All interferon was administered i.m. Three additional African green monkeys received the same regimen with interferon alpha alone first, followed by interferon gamma alone, and 5 days of combination therapy as described above. There were no significant blood chemistry or urinalysis changes attributed to interferon therapy. The average peak levels of interferon achieved and the time after injection of peak concentration are given in table 1.

The data are being analyzed further to see if apparent accumulation during the combination therapy is statistically significant.

The combination therapy provides serum levels of interferon that should be effective without causing any significant perturbation in clinical chemistry or pyrexia (5). Infected animals will be treated with alpha and gamma combinations to test for efficacy.

7. <u>Intrathecal administration of ribavirin and other antivirals</u>. Ribavirin was administered intrathecally (i.t.) to monkeys at the following dosages daily for 5 days: 0.05 mg/kg, 0.5 mg/kg, 2.0 mg/kg, 5.0 mg/kg, and 25 mg/kg. All monkeys experienced a mild, reversible anemia that can be attributed solely to the number of blood samples taken, since the same anemia was present in the control monkey treated i.t. with Hank's Balanced Salt Solution (HBSS). Monkeys treated with 0.05 and 0.5 mg/kg showed no ill effects of treatment. Monkeys treated with 2.0 and 5.0 mg/kg showed a reversible depression of appetite on days 2, 3, 4, and 5 of treatment. One dose of 25 mg/kg ribavirin proved fatal. Serial serum and CSF samples are being processed by high performance liquid chromatography to determine i.t. treatment pharmacokinetics. Two monkeys received 3 mg/kg i.t. ribavirin triacetate under the same treatment schedule with no contraindications.

PRESENTATIONS:

1. **Kende, M.** Efficacy of muramyl tripeptide against Rift Valley fever and herpes simplex virus infections. Presented, Ciba-Geigy, Basel, Switzerland, 1985.

2. **Kende, M.** Prophylactic and therapeutic efficacy of poly(ICLC) against Rift Valley fever virus infection in mice. Presented, Conference on Polyribonucleotides for Cancer Therapy, NIH, Bethesda, MD, 1985.

3. **Canonico, P.** Approaches to combination chemotherapy. Presented, Antiviral workshop - NIAID, NIH, Bethesda, MD, October 10, 1985.

PUBLICATIONS:

1. **Kende, M., J. E. Talmadge, W. R. Rill, W. Pannier, and P. G. Canonico.** 1984. Therapy of Rift Valley fever virus infection with poly(ICLC) and ribavirin, pp. 205. *In* abstracts of the 24th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, DC.

2. **Canonico, P. G., and P. B. Jahrling.** 1985. Chemotherapy for "exotic" RNA viruses. *J. Antimicrob. Chemother.* 15:129-138.

3. **Canonico, P.G.** 1985. Efficacy, toxicology and clinical applications of ribavirin against virulent RNA viral infections. *Antivir. Res.* (In Press).

LITERATURE CITED

1. **Huggins, J. W., P. B. Jahrling, M. Kende, P. G. Canonico.** 1984. Efficacy of ribavirin against virulent RNA virus infections, pp. 49-63. *In* R. A. Smith, V. Knight, J. A. D. Smith (ed.), Clinical applications of ribavirin, Academic Press, New York.

2. **Peters, C. J.** 1984. Arenaviridae, pp. 513-545. *In* R. E. Belshe (ed.), Textbook of human virology. P. S. G. Littleton.

3. **Maiztegui, J. I.** 1975. Clinical and epidemiological patterns of Argentine hemorrhagic fever. *Bull. WHO.* 52:576-676.

4. **Hopkins, A.** 1983. Survival analysis with covariates - Cox models, pp. 576-594. *In* W. J. Dixon, (ed.), BMDP statistical software. University California Press.

5. **Levy, H. B., and F. L. Riley.** 1983. A comparison of immune modulating effects of interferon and interferon inducers, pp. 303-322. *In* M. Landy (ed.), Lymphokines. Academic Press, New York.

Table 1. Average responses of three monkeys receiving alpha interferon alone, then 5 days of combination therapy

Treatment	Alpha µg/ml Concentration	Peak Time After Injection (min)	Gamma	Peak Time After Injection (min)
alpha	480	240	0	-
gamma	0	-	5.2	360
alpha + gamma day 1	480	360	6.5	360
alpha + gamma day 5	780	480	12.2	30
gamma	-	-	25.0	360
alpha	850	360	-	-
alpha + gamma day 1	1700	480	15.0	360
alpha + gamma day 5	1400	180	16.0	30

Table 2. Incremental relative risk of death with 20 µg Poly(ICLC) regimens vs. standard (day 0) treatment of Rift Valley fever virus-infected, Swiss Webster Mice (N=10)

TREATMENT ON DAY (S)			NO. OF SURVIVORS	RELATIVE RISK OF DEATH	P VALUE
+3			0	38.07	< 0.05*
+2			0	21.19	
-5			0	7.81	
	-4 +4		0	6.42	
-3			0	4.96	
	-5 +5		1	4.47	
-4			1	4.33	
	-3 +3		0	4.12	
-2			1	2.69	
+1			1	2.39	≥ 0.05**
	-2 +3		2	1.35	
	-3 +1		2	1.14	
	-2 +2		3	1.05	
0	(STANDARD TREATMENT)		3	1.00	
-1			4	0.92	
		-1 +6 +11	7	0.39	
	-1 +4		8	0.36	
		-2 +2 +4	8	0.22	
	0 +4		8	0.21	< 0.05***
		-3 +1 +5	8	0.21	
		-1 +1 +3	9	0.11	
		-4 +1 +6	9	0.11	
		0 +4 +7	9	0.10	
	-1 +1		10	UNDEFINED	

P<0.05*= More animals at risk than with standard treatment.

P≥0.05**= Indistinguishable.

P<0.05***= Less animals at risk.

________=Significantly less at risk.

Challenge dose: 250 PFU.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMB
				DAOB 6410	85 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISS'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	H. TERM	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763A	3M263763D807	AE	015
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Development of Therapeutic and Prophylactic Regimens in Man

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0601 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	85 10	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	1.0	339
c. TYPE	d. AMOUNT	86	0.0	0*
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Medical Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Hammond, J B
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7655
21. GENERAL USE FINA; MILITARY/CIVILIAN APPLICATION: M	f. NAME OF ASSOCIATE INVESTIGATOR (if available)
	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Vaccines; (U) Volunteers; (U) High Containment Medical Care

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Evaluate experimental vaccines developed by USAMRIID, various contractors, organizations, and other government agencies; assess differences in immune responses utilizing computer techniques to analyze a vast database; compare interlot differences in immune response; evaluate new vaccine lots and conduct field trials; maintain research data relevant to medical defense against potential BW agents and other infectious agents of unique military importance. Maintain facilities to transport and treat patients under conditions of total (P-4) biohazard containment.

24. (U) Experimental vaccines which have undergone safety testing in animal models and have been approved after strict protocol testing and extensive scientific and ethical reviews are administered to human volunteers. Immune responses are measured by conventional serologic and lymphocyte assays and skin testing. Results are correlated with immunization schedules.

25. (U) 8410-8509-The most significant accomplishment in 1985 was the elimination of the backlog in annual reports to the FDA on experimental vaccines. Another significant accomplishment was further refinement in the computer program for entry of vaccination data. The maximum containment facilities in Medical Division have been further upgraded in terms of equipment, and improvements have been made in the ongoing training program of assigned personnel.

*This work unit has been suspended (unfunded) for FY 86.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AE-015: Exploratory Development of Therapeutic and Prophylactic Regimens in Man

PRINCIPAL INVESTIGATOR: J.B. Hammond, M.D., COL, MC

ASSOCIATE INVESTIGATORS: None

Background:

Special Immunizations Program: In 1981, the Department of Occupational Medicine was formed within the Medical Division to provide a formal and closely supervised approach to managing the USAMRIID Special Immunizations Program. A physician was placed in charge. The purpose of this program is to administer both investigational and licensed vaccines to personnel at risk of exposure to selected infectious agents and to monitor the serological responses.

Maximum Containment Facilities: The Medical Division maximum containment facilities, consisting of both stationary and aeromedical units, provide the capability for care and transport of patients exposed to highly infectious or toxic agents. The need for these facilities arises from the mandate to support the overall USAMRIID research effort by providing patient care to personnel presumed to have been exposed, or known to have been exposed, to such agents. The facilities provide a unique national resource, in that a soldier, a researcher, or any designated individual with exposure to highly infectious or toxic agents can be transported under maximum containment conditions to USAMRIID from virtually any site worldwide and cared for under the same conditions.

Summary:

Special Immunizations Program: The objectives set in the last four years for the Special Immunizations Programs have been to bring organization to the day-to-day operations of the Program (utilizing computer techniques); to continue verification of immunization requirements through communication with division chiefs; to improve the timeliness of initial and booster vaccinations; to analyze responses to vaccines; to improve the quality of annual reporting to the Food and Drug Administration (FDA); to provide better educational services to Fort Detrick Facilities Engineering personnel enrolled in the Program; to ensure that data collected via vaccine safety and efficacy trials are added to the vaccine database; and to develop computerized profiles on USAMRIID personnel, incorporating vaccination and medical surveillance data.

Maximum Containment Facilities: Clinical Research Ward personnel associated with the ward maximum containment facilities are given a formalized quarterly course of orientation and instruction. The objectives are twofold: orientation of newly assigned personnel in an environment of rapid turnover, and periodic review for other personnel to ensure continued readiness.

Physicians from the WRAMC Infectious Disease Service, Department of Surgery, and intensive care units are scheduled to begin participation in the quarterly training sessions on the care of patients under high containment conditions.

The members of the Aeromedical Isolation Team participate in bi-monthly in-house training to maintain skills for transport and care of patients under high containment conditions. Joint cross-country in-flight training exercises are coordinated between the team and the 167th West Virginia Air National Guard.

Progress:

Special Immunizations Program: Utilizing computer programs, efforts to improve the quality of FDA reports have led to preliminary analysis of vaccine responses to selected vaccines. Extensive analysis awaits the assurance that all data in the database are correct. This verification step is presently near completion.

The special immunizations nurse now enters vaccine data in the computer the day they are generated and computer entries are verified weekly. This ensures that data are always current and facilitates generation of immunization profiles. Verification of computer data collected over the past decade with hand-written records has also been completed and ensures that computer immunization histories are correct. Steps are now being taken to ensure that vaccination data generated by various protocols conducted in the past are complete and in the computer. The important goal of eliminating the backlog in annual reports on vaccine IND's to the FDA has been accomplished.

Maximum Containment Facilities: The Clinical Research Ward High Containment Unit was utilized in May to provide evaluation and patient care to a USAMRIID worker involved in a laboratory accident. The worker was suspected of having been exposed to Junin virus. Patient care was provided over five days by the Clinical Research Ward staff. The worker was released after investigation of the accident led to the conclusion that there was low risk of actual exposure to Junin virus.

PUBLICATIONS: None

PRESENTATIONS: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 302666	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	H. TERM	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763D	3M263763D807	AF	016
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82-83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Development of Vaccines Against Argentine Hemorrhagic Fever

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0603 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	85 10	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	4.4	958
c. TYPE	d. AMOUNT	86	0.0	0*
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Lupton, H W
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2405
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Barrera-Oro, J
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Cole, F E, Jr

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Junin Virus; (U) Military Medicine; (U) Viral Diseases; (U) Prophylaxis; (U) Lab Animals; (U) BW Defense; (U) RAM I; (U) Monkeys

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop and produce live-attenuated Junin vaccine which may be administered to at-risk military personnel for prophylaxis in geographical endemic areas.

24. (U) Junin virus will be propagated in certified diploid cell cultures, cloned, and selected for markers-of-attenuation. Resultant vaccine seeds and vaccine will be safety tested to assure freedom from contamination or adventitious agents. Vaccine efficacy will be estimated in animal models by determination or serological conversion and protection against virulent challenge.

25. (U) 8410-8509-Histological, "blind" study of CNS lesions from Junin-infected and control rhesus monkeys showed minimal lesions, variable in extent. In juvenile monkeys, vaccine virus was recovered from the spleen of one and liver of another and 2 weeks post-infection, but no viral antigen was detectable by FA. No virus was found in the organs of these or other animals up to 8 weeks post-vaccination. Differences between control and vaccinated animals were minimal and not statistically significant. A nominally acceptable, freeze-drying menstruum was developed for vaccine production. An IND submission was made to the FDA.

*This research will be part of Work Unit 807AC in FY 86.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AF-016: Exploratory Development of Vaccines Against Argentine Hemorrhagic Fever

PRINCIPAL INVESTIGATOR: Harold W. Lupton, COL, Ph.D.

ASSOCIATE INVESTIGATORS: F. E. Cole, Jr., Ph.D.
J. G. Barrera Oro, Ph.D.
A. Kuehne, CPT, Ph.D.

Background:

Studies have continued on the development of an attenuated Argentine hemorrhagic fever (AHF) vaccine. Junin virus (JV), a member of the arenavirus family, causes an acute, severe, and moderately lethal disease (ca. 15% of cases). Studies on vaccine development for this disease are clearly beneficial to the US Army for several reasons, *viz*: a) the vaccine thus developed may be used to immunize US troops and/or at-risk personnel; b) only very limited vaccine experience is available with any arenavirus (Junin, Clone 3, 1970), and, since no other agency or commercial firms have initiated such studies, we are thus compelled to do so simply to protect our Army personnel, regardless of how they may become exposed; c) we desperately need to gain experience with arenavirus vaccine development in general, since other related viruses that cause hemorrhagic fevers have been described in Bolivia (BHF) and Africa (Lassa); and, d) the active presence of other, but nonpathogenic, arenaviruses in the USA, Trinidad, Colombia, Brazil, and Paraguay herald the possibility that still more unknown arenavirus-caused hemorrhagic fever outbreaks may emerge in the immediate future, a possibility for which we can be prepared by virtue of studies such as this one.

As previously described (1), three final candidates were selected for further evaluation as vaccine candidates. All three were more attenuated than even the Argentinian Clone 3 vaccine, when tested in 11- to 12-day-old mice, adult guinea pigs, and baby guinea pigs, (all i.c.). Also, they were all shown to be genetically stable during multiple cell culture passage. One of them, Candid #1, also demonstrated little or no neurovirulence when tested in guinea pigs (1). This virus, Candid #1, was taken to final product in 1982 (2), by methods approved for Biologics (3), and under GLP Regulations (4).

In-process testing required by the FDA was initiated in 1982 and with the exception of final container tests, was completed in 1983 (5). During the present reporting period, studies initiated in FY 84 (6) were continued on the development of an optimal freeze-drying menstruum. Additional studies in FY 84 (6) included a long-term (7-1/2 month) evaluation of Candid #1 in rhesus monkeys; this demonstrated that the new Junin vaccine elicited only minimal lesions in the CNS of 50% of test animals. The study further showed that no active necrosis of neurons or neurological cells occurred in these animals. Even fewer monkeys (ca. 17%) showed any minor visceral lesions that might be vaccine-associated. Significantly, no virus was isolated from the CNS, spleen, lymph nodes, liver, pancreas, lung, kidney, or brown fat of these monkeys.

Summary:

A 7-1/2-month study in rhesus monkeys given Candid #1, live, attenuated Junin virus vaccine (see AR FY84) revealed no virus in the CNS or viscera of vaccinated animals, as determined by co-cultivation. However, histological examination showed minimal CNS lesions without neuronal involvement in 6 of 12 monkeys studied. To disprove any possibility of bias in describing these lesions, similar sections were obtained from the same tissue blocks and examined in a "blind" study by two independent pathologists. Again, minimal lesions were found by both examiners; however, lesions were described in both control and vaccinated monkeys. The two pathologists reported varied results on the extent of the affected regions.

The vaccine study was repeated in a new group of juvenile (1-to-2-year-old) monkeys to permit evaluation of neurological tissues at earlier and later periods post-inoculation. Junin vaccine virus was recovered from the spleen of one monkey and the liver of another that were killed 2 weeks after inoculation, although no viral antigen was detectable in those organs by the fluorescent antibody (FA) technique. Other organs of these two animals, as well as all the organs of those animals killed 4 and 8 weeks after vaccination, were negative for virus, even though all vaccinated animals seroconverted to Junin virus. "Blind" histologic examination of rhesus that were killed 2, 4, and 8 weeks after vaccination, again revealed minimal CNS lesions without neuronal involvement in vaccinated (11/12) and control (5/6) rhesus. Although differences between vaccinated and control animals were minimal and not statistically significant, there was a suggestion of a Candid #1 virus-related increase in basal CNS lesions.

Because neither infectious virus nor virus antigen was found in the CNS of vaccinated rhesus, we cannot ascertain whether the minimal lesions are related to the presence of Junin virus in the CNS; if they enhanced pre-existing lesions; or if these lesions were related to the age of the rhesus.

Continued efforts to develop an optimal freeze-drying menstruum for the Candid #1 vaccine have resulted in a nominally acceptable combination that will be used for the first small-scale lots. Work will continue in a joint USAMRIID/Salk Institute venture to formulate the optimal menstruum.

Progress:

Additional CNS sections from rhesus given Candid #1 vaccine (6) in the previous long-term study were prepared by cutting blocks 100 μm deeper than the one examined in FY84. The sections then were examined in a blind study by two pathologists (G,H). Table 1 is a summary of the results obtained in the current study and in the previous CNS examination by pathologist G. Minimal CNS lesions without neuronal involvement were described by both pathologists in vaccinated and control rhesus. Pathologist G described lesions in two out of five control monkeys (in 0/6 controls in FY 84), in four out of six monkeys given vaccine diluted 1/10 (in 4/6 in FY 84) and in four out of six given undiluted vaccine (2/6 in FY 84). Pathologist H described lesions in four out of five control animals, in three out of six animals given vaccine diluted 1/10, and in five out of six animals given undiluted vaccine. Thus, in relation to the number of rhesus with CNS lesions, neither pathologist found statistically significant differences between control and vaccinated animals. Moreover, pathologist H described equal numbers of CNS regions affected in control and vaccinated animals and consequently the corresponding neurovirulence scores were virtually the same for control rhesus (score: 1.2), for rhesus given 4 x 10^4 PFU of vaccine (score: 1.2), and for rhesus given 4 x 10^5 PFU of vaccine (score 1.0). Pathologist G, however, described twice as many affected CNS regions in animals receiving 4 x 10^4 PFU of vaccine than in controls and three times more affected CNS regions in animals receiving 4 x 10^5 PFU of vaccine than in controls, with corresponding neurovirulence scores of 0.6, 1.2, and 1.7 respectively. (See Table 1).

A complementary study was conducted to try to ascertain whether the minimal CNS lesions reported in the previous (FY 84) study represented the early stages of late occurring lesions or were the terminal stages of earlier, transient lesions. Eighteen captive-bred rhesus monkeys, 1- to 2- years-of age, were randomized into two groups. Group 1 (12 rhesus) received 2.6 x 10^4 PFU of Candid #1, i.m., in 0.5 ml (the dose that was associated with more minimal CNS lesions upon first examination of sections in the FY 84 study). Group II (6 monkeys) received 0.5 ml of saline, i.m. The animals were observed daily for neurological signs of disease: head tilt; sagging and/or drooping eyelid, lip, cheek; circling in cage; head pressing; ataxia; muscle tremors and/or fasciculation; muscle atrophy; paralysis; nistagmus; strabismus; myosis; mydriasis; anisocoria; personality change (more or less aggressive); unconsciousness; and general clinical signs of disease. Neutralization tests against Junin virus were carried out with paired serum samples of the animals to demonstrate lack of Junin virus infection in controls and successful virus infection of the vaccinated rhesus. Two vaccinated and one control rhesus were killed 2 weeks after inoculation; five vaccinated and two controls were killed 4 weeks after inoculation; five vaccinated and three controls were killed 8 weeks after inoculation; and five vaccinated and three controls will be killed 51 weeks after vaccination. The killed animals were examined for virus by co-cultivation of spleen, liver, lymph node, salivary gland, frontal cortex, temporal cortex, occipital cortex, thalamus, pons, medulla, cerebellum, and lumbar spinal cord and for Junin virus antigen by direct immunofluorescence of the previously described organs.

Gross and histologic examination of killed monkeys was carried out in a "blind" fashion (the identity of control and vaccinated animals was not known by the pathologist until after preparation of draft reports on all of the animals). Daily observation of the animals revealed: restlessness in two control rhesus (similarly the animal observers did not know the identity of control and vaccinated animals); loss of hair (scabies) in two control and two vaccinated animals; diarrhea in two vaccinated monkeys (some animals had been infected with *Trichiuri trichiura* before the initiation of the study). No signs attributable to CNS problems were observed in vaccinated rhesus. That all Candid #1 vaccine-inoculated rhesus seroconverted to Junin virus is shown in Table 2. No Junin virus antigen was detected in any of the organs of the animals that were killed 2, 4, and 8 weeks after inoculation, although Junin virus was cocultivated from liver of 1 of 2 and spleen of 1 of 2 rhesus killed on the second post-inoculation week. Co-cultivation of the other organs of the two vaccinated monkeys that were killed 2 weeks after inoculation and of all the organs of the animals killed on post-vaccination weeks 4 and 8 gave negative results. "Blind" histologic examination of the animals revealed background CNS lesious in 5 of 6 controls (Table 3); 7 of 12 vaccinated rhesus also showed CNS lesions within background limits; 3 of 12 vaccinated rhesus showed lesions in 3 of the 16 CNS regions examined and were considered suspicious but inconclusive evidence of neurotropism by the pathologist. He indicated that one rhesus killed 4 weeks post-inoculation (rhesus 2R9) had lesions in 8 different regions of the nervous system. These lesions were distributed in a similar pattern to that observed as a result of virulent Junin virus infection. All eight lesions in this rhesus were considered minimal; no degenerate or necrotic neurons were observed in the nervous systems of this rhesus. The pathologist clearly indicated in the final report that no neuronal changes were seen in any principal or control animal in the study and that he suspects that the candidate vaccine retains some very minimal neurotropism, but that in the absence of neuronal changes in all animals, the histologic lesions are unlikely to have any clinical significance. Five vaccinated rhesus and three controls will be killed and studied approximately 1 year after inoculation.

Efforts to improve the stabilizer for the dry Candid #1 vaccine continued in FY 85 in cooperation with The Salk Institute-Government Services Division, Swiftwater, PA. Thirty-one trial products, obtained in nine drying cycles, were assayed for virus recovery from the dry product and temperature stability of the dry and reconstituted product. Summarized in Tables 4, 5, 6, 7, and 8 are the comparative effects of different components on drying and stability of Junin Candid #1 virus. After a careful review of all the data relative to titer loss during freeze-drying, total loss, loss after reconstitution, and loss in dry form at 35°C in addition to osmolarity, an interim stabilizer was selected. The chosen stabilizer, containing human serum albumin, sorbitol, sodium glutamate, and degraded gelatin in the right concentration to obtain a physiologic osmolarity after reconstitution and a pH of 7.0 to 7.1, was selected for the present batch of the Candid #1 vaccine because it had produced the highest percentages of virus recovery in the dry product. This stabilizer has also given reproducible results in several drying trials, including one in which a semi-mass scale operation was mounted, and has maintained acceptable titers at -20°C for almost 1 year (1 month = 76% recovery; 16 months = 38-43%; 20 months = 36%). Neither the chosen stabilizer, nor the almost 100 other stabilizers that have been tested so far have enhanced the heat stability of the product. Thus, the dry vaccine must be stored and transported at -20°C and must be used within 4 h after reconstitution, if held > 20°C. Clearly, studies directed towards the development of more suitable stabilizer(s) must continue in a joint USAMRIID/Salk Institute venture.

PRESENTATIONS:

1. **McKee, K. T. Jr., J. G. Barrera Oro, A. I. Kuehne, J. Spisso, and B. G. Mahlandt.** Immunogenicity and protective efficacy of a live attenuated Argentine hemorrhagic fever (AHF) vaccine in primates. Presented, XI International Congress for Tropical Medicine and Malaria, Calgary, Canada, 16-22 Sept 1984.

2. **Lupton, H. W., F. E. Cole, Jr., E. D. Green, K. T. McKee, Jr., J. Donovan, A. I. Kuehne, G. French, K. Johnson, G. Eddy, and J. G. Barrera Oro.** Safety and efficacy of a live attenuated vaccine against Argentine hemorrhagic fever. Presented, 6th International Congress of Virology, Sendai, Japan, 20-23 Sept 1984.

3. **Barrera Oro, J. G., K. T. McKee, Jr., A. I. Kuehne, J. Spisso, and B. G. Mahlandt.** Recent progress in testing a live, attenuated Argentine hemorrhagic fever (AHF) vaccine. Presented, Annual Meeting of the American Society of Tropical Medicine and Hygiene, Baltimore, Maryland, 2-6 Dec 1984.

4. **Barrera Oro, J. G., K. T. McKee, Jr., A. I. Kuehne, J. B. Moe, D. E. Green, F. E. Cole, Jr., and H. W. Lupton.** Preclinical trials of a live, attenuated Junin virus vaccine in rhesus macaques. Presented, Symposium on Vaccines and Vaccinations, Paris, France, 4-7 June 1985.

PUBLICATIONS: None

LITERATURE CITED

1. U.S. Army Medical Research Institute of Infectious Diseases, 1 Oct. 1981. Annual Progress Report, FY 1981. Ft. Detrick, Maryland.

2. U.S. Army Medical Research Institute of Infectious Diseases, 1 Oct 1982. Annual Progress Report, FY 1982, Ft. Detrick, Maryland.

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TABLE 1. "Blind" re-examination of rhesus central nervous system tissues from study Junin 83-1

Inoculum	Rhesus		Pathology: Rhesus with positive or negative CNS lesions, Pathologist "G"	Pathology: Rhesus with positive or negative CNS lesions, Pathologist "H"	Number of CNS regions showing lesions, Pathologist "G"	Number of CNS regions showing lesions, Pathologist "H"	Neurovirulence scores[a], Pathologist "G"	Neurovirulence scores[a], Pathologist "H"
	86R		- (-)[b]	+	0 (0)[b]	1		
	118R		+ (-)	+	2 (0)	3		
Saline (controls)	226S		ND (-)	+	ND(0)	1	0.6 (0.0)[b]	1.2
	B67		- (-)	ND	0 (0)	ND		
	210R		+ (-)	+	1 (0)	1		
	198R		- (-)	-	0 (0)	0		
		Total:	2/5 (0/6)	4/5	3 (0)	6		
	36S		+ (+)[b]	+	3 (4)[b]	3		
	251S		+ (+)	+	1 (2)	1		
Vaccine (4×10^4 PFU)	297R		- (-)	-	0 (0)	0	1.2 (2.3)[b]	1.2
	286R		+ (+)	+	1 (2)	3		
	19S		- (-)	-	0 (0)	0		
	057S		+ (+)	-	2 (6)	0		
		Total:	4/6 (4/6)	3/6	7 (14)	7		
	166R		+ (+)[b]	+	4 (1)[b]	2		
	129R		- (-)	-	0 (0)	0		
Vaccine (4×10^5 PFU)	117S		- (-)	+	0 (0)	1	1.7 (0.6)[b]	1.0
	208S		+ (-)	+	2 (0)	1		
	154S		+ (+)	+	3 (3)	1		
	3S		+ (-)	+	1 (0)	1		
		Total:	4/6 (2/6)	5/6	10(4)	6		

[a]WRAIR scoring system (No. and severity of CNS regions affected/No. of animals)
[b]Results of first examination in 100 μm shallower sections

TABLE 2. Seroconversion to Junin virus in juvenile rhesus macaques inoculated with Candid #1 Junin virus vaccine or saline (Study Junin 84-1).

Inoculum	Rhesus No.	Junin virus neutralizing antibody titer[a] (day)	
		Prevaccination	Postvaccination
Saline (control)	2R8	-[b] (-6)	- (14)
	82D44	- (-6)	- (28)
	2N8	- (-6)	- (29)
	2N24	- (-6)	- (56)
	82D34	- (-6)	- (57)
	2N38	- (-6)	- (58)
Candid #1 Vaccine 2.6×10^4 PFU	2R6	- (-6)	8 (14)
	2N15	- (-6)	32 (14)
	2R2	- (-6)	128 (28)
	2N27	- (-6)	32 (28)
	2R9	- (-6)	64 (29)
	82D53	- (-6)	256 (29)
	2N40	- (-6)	64 (29)
	82D38	- (-6)	4096 (56)
	2N25	- (-6)	1024 (57)
	2N34	- (-6)	4096 (57)
	2N33	- (-6)	64 (58)
	82D61	- (-6)	1024 (58)

[a] Expressed as reciprocal of serum dilution causing PRN_{80} of XJ Clone 3.
[b] - = Negative.

TABLE 3. Reconstructed results of "blind" histologic examination of rhesus CNS from Junin 83-1 and Junin 84-1[a].

Time after inoculation	Rhesus with CNS lesions/total		CNS regions with lesions			Neurovirulence scores	
	Vaccine	Controls	Vaccine	Controls		Vaccine	Controls
2 weeks[b]	2/2	1/1	4	2		2.0	1.0
4 weeks[b]	4/5	2/2	14	2		2.8	1.0
8 weeks[b]	5/5	2/3	11	2		2.2	0.7
32-34 weeks[c]	4/6	2/5	7	3		1.2	0.6
Total:	15/18	7/11	36	9	Average:	2.0	0.8

[a] This table was constructed from results of "blind" examinations conducted by Pathologist G of CNS tissue from monkeys given Junin vaccine diluted 1:10.

[b] Study Junin 84-1: Vaccine 1/10 = 2.6×10^4 PFU.

[c] Study Junin 83-1: Vaccine 1/10 = 4.0×10^4 PFU.

TABLE 4. Effect of sorbitol, degraded gelatin, peptone, lactose, pH, and Na and K glutamate on freeze-drying and stability of Junin Candid #1 virus.

Item	Trial Number						
	8	9	10	11			
				A	B	C	D
Initial virus dilution	1:15	1:10	None	1:6.25			
Virus:stabilizer ratio	1:1	1:1	1:12.5	1:1			
Lactose (final), %	5	7	7	7			
Glutamic acid (final), %	1	2	0.9	2	1	2	1
HSA (final), g%	2.5	2.025	2.5	2.1			
Osmolarity, (MosM/kg)	392	460	301	488	414	513	394
Fill, ml	1.2	1.2	5.5	5.5			
Post-dry pH	7.4	6.3	6.0/7.0	6.7	6.9	6.7	7.0
Av. Residual moisture, %	1.6	1.6	1.1	2.4			
Loss in drying, %	15-16	5-13	61-62	24-37			
Loss reconstituted 5 hrs at 4°C, %	N.T.[a]	N.T.	N.T.	23-63			
Loss 35°C/48 h, %	70-83	N.T.	N.T.	78-92			
Control or variable tried	Sorbitol Degraded gelatin	Peptone Lactose Vac/N_2	Two pH levels	Alterration of acids Na/K)			

[a]N.T. = Not Tested.

TABLE 5. Effect of sorbitol, Na glutamate, PO_4, arginine, and PO_4/arginine on freeze-drying and stability of Junin Candid #1 virus.

Constituents used for drying	Constituents included by test[a]					
	13-1	13-2	13-3	13-4	13-5	13-6
Hydrolyzed gelatin 2%	X[b]	X	X	X	X	X
Sorbitol, 7.5%	X					
Sucrose, 7.5%		X	X	X	X	X
Na/K glutamate, 3%	X	X		X	X	X
Na glutamate, 3%			X			
PO_4 buffer, 0.025M				X		X
L Arginine HCl, 1%					X	X
Phenol red, 0.0005%	X	X	X	X	X	X
Osmolarity-drying	764	628	640	710	705	781
Osmolarity-reconstituted	374	314	309	349	343	387
Effect measured	Sorbitol	[c]	Na glutamate	PO_4	Arginine	PO_4/arg
Loss in drying, %	25	29	20	19	31	24
Total loss[d], %	13	29	29	14	24	11
Loss 4°C 2 wks, %	38	40	18	52	34	48
Loss 4°C reconst. 5 h, %	31	21	15	37	25	41
Loss 35°C 48 h, %	77	92	88	86	84	81

[a]Parameters common to all six tests were:
Virus dilution: 1:6.25 (2X) Dried atmosphere: dry nitrogen
Fill volume: 2.75 ml/20 ml vial Overall moisture: 1.9%.
[b]Indicates constituents included in a given freeze-dry run (#1-#6).
[c]This stabilizer was used in previous trials and was used for comparison.
[d]From calculated original bulk titer.

TABLE 6. Effect of arginine with different combinations of trehalose or sucrose on the freeze-drying and stability of Candid #1 Junin virus.

Constituents used for drying	Constituents included by test			
	14-1	14-2	14-3	14-4
Hydrolyzed gelatin, 2%	X[a]	X	X	X
Trehalose, 5%	X			
Trehalose, 7.5%			X	
Trehalose, 10%		X		
Sucrose, 5%	X			
Sucrose, 7.5%				X
Na/K glutamate, 3%	X	X	X	X
PO_4 buffer, 0.025 M	X	X	X	X
Arginine HCl, 1%	X	X	X	X
Phenol Red, 0.0005%	X	X	X	X
Osmolarity reconsituted (2X)	423	391	357	383
Effect measured: arginine w/different combinations of trehalose vs sucrose				
Loss in drying, %	0	13	12	8
Total loss[b], %	24	32	30	26
Loss 4°C reconstituted 5 h, %	16	13	13	24
Loss 35°C 48 h, %	90	90	92	90

[a]Indicates constituents included in a given freeze-dry test.
[b]Calculated from original bulk titer.

TABLE 7. Effect of gelatin, sol-u-pro, urea/allantoin/lactose, and trehalose glycerol on the freeze-drying and stability of Junin Candid #1 virus.

Constituents used for drying	Constituents included by test							
	15-P	16-P	15-A	16-A	15-B	15-C	15-D	15-E
Degraded gelatin. 2.5%	X[a]	X						
Sol-U-Pro, 2.5%			X	X				
Sorbitol, 5.0%	X	X	X	X				
Sodium glutamate, 2.0%	X	X	X	X				
Urea, 2.0%					X	X		
Allantoin, 0.2%					X	X		
Lactose, 5.0%					X	X		
Citric acid, 0.2%						X		
Trehalose, 5.0%							X	X
Glycerol, 2.0%							X	X
Ascorbic acid, 0.2%								X
Phenol red, 0.0005	X	X	X	X	X	X	X	X
Osmolarity, reconstituted	271	271	270	270	266	283	242	255
pH, reconstituted	6.0	7.0	6.0	7.0	7.1	7.0	7.3	7.2
Effect measured:	gelatin vs. sol-U-Pro				urea-Allan lactose		trehalose glycerol	
Loss in drying, %	16	36	33	11	ND	21	0	73
Total loss[b], %	16	36	38	17	24	33	10	80
Loss 29°C, reconstituted 5 h, %	59	44	45	43	62	49	37	81
Loss 35°C 48 h, %	80	81	68	78	58	54	90	100

[a] Indicates constituent included in a given freeze-dry trial-test.
[b] Computed from titer of original bulk virus.

TABLE 8. Effect of gelatin/sorbitol/glutamate, urea/allantoin, and gelatin/lactose on the freeze-drying and stability of Junin Candid # 1 virus.

Constituents used for drying	Constituents included by test					
	17-A	17-B	17-C	17-D	17-E	17-F
Sol-U-Pro, 2.5%	X[a]		X	X	X	X
Sorbitol, 5.0%	X					
Monosodium glutamate, 2.0%	X					
Urea, 2.0%		X	X			
Urea, 3.0%				X	X	
Allantoin, 0.2%		X	X			
Allantoin, 0.3%				X	X	
Lactose, 5.0%		X	X	X	X	X
Phenol red, 0.005%	X	X	X	X	X	X
Effect measured: gelatin-sorbitol-glutamate/urea-allantoin or gelatin-lactose						
Loss in drying, %	27	19	24	34	13	11
Total loss[b], %	28	47	47	43	45	39
Loss 29°C reconstituted 5 h, %	26	56	32	44	40	64
Loss 35°C 48 h, %	72	61	56	75	74	92

[a] Indicates constituent included in given freeze-dry trial test.
[b] Computed from titer of original bulk virus.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 302669
2. DATE OF SUMMARY: 85 10 01

REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 84 10 01
4. KIND OF SUMMARY: D. CHANGE
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES:

	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763D	3M263763D807	AH	017
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code): Exploratory Development for Rapid Identification and Diagnosis

12. SUBJECT AREAS: 0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE: 83 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE: | EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE: | d. AMOUNT:
e. KIND OF AWARD: | f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	2.3	439
86		

19. RESPONSIBLE DOD ORGANIZATION

a. NAME: USA Medical Research Institute of Infectious Diseases
b. ADDRESS (include zip code): Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL: Huxsoll, D L
d. TELEPHONE NUMBER (include area code): 301-663-2833

20. PERFORMING ORGANIZATION

a. NAME: Disease Assessment Division, USAMRIID
b. ADDRESS: Fort Detrick, MD 21701-5011
c. NAME OF PRINCIPAL INVESTIGATOR: LeDuc, J W
d. TELEPHONE NUMBER (include area code): 301-663-7244
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Meegan, J M
g. NAME OF ASSOCIATE INVESTIGATOR (if available):

21. GENERAL USE: FIC

MILITARY/CIVILIAN APPLICATION: M

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Bacterial Diseases; (U) Immunology; (U) Diagnosis

23. TECHNICAL OBJECTIVE 24. APPROACH 25 PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop, test, and perfect assays to rapidly detect and identify biological agents obtained from environmental samples or infected military personnel.

24. (U) Antigen and early (IgM) antibody-capture enzyme immunoassay technology has been the approach adopted.

25. (U) 8410-8509-Rapid (3-hr), simple antigen and antibody detection assays developed in previous years have been adapted and perfected. Monoclonal antibodies against sandfly fever virus (Sicilian) have been produced, characterized, then optimized for use in rapid assays. Monoclonal antibodies have been acquired and employed to improve sensitivity and specificity of assays for Rift Valley fever, Crimean-Congo hemorrhagic fever, Hantaan (Korean hemorrhagic fever), and Chikungunya viruses. Validation of each assay with field-collected, epidemiologically relevant samples has continued. The inactivation of certain biohazardous viruses used as antigens in these assays has been studied. Under specific controlled conditions, both cobalt and β-propiolactone were found to inactivate Hantaan virus while retaining antigenicity. Additionally, these types of assays were studied to determine the amount of antigen present in various lots of vaccine. Preliminary results indicate these assays might be useful in replacing animal inoculation tests to predict the potency of vaccines under development.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AH-017: Exploratory Development for Rapid Identification and Diagnosis

PRINCIPAL INVESTIGATOR: James W. LeDuc, LTC, Ph.D.

ASSOCIATE INVESTIGATOR: James M. Meegan, CDR, USN, Ph.D.

Background:

The goal of the Rapid Diagnosis Section is to develop rapid, simple tests for identification of biological agents. It is anticipated that samples containing unknown agents will be received from both environmental and clinical sources. The main effort has been to detect antigen, although it should be emphasized that infected servicemen might be the first sign of the presence of a disease-causing agent. Consequently, for each agent, an attempt has been made to develop not only a rapid antigen-detection test, but also a rapid immunoglobulin M (IgM)-detection assay to measure antibodies in a single serum sample collected early in clinical disease but after the period of antigen circulation. Identification of biological warfare agents generally encompasses two categories of specimens: environmental and clinical.

In previous years, rapid assays for the detection of antigen in clinical or environmental samples were developed for Rift Valley fever, sandfly fever (Sicilian), Venezuelan equine encephalomyelitis, Crimean-Congo hemorrhagic fever, West Nile, Chikungunya, and Sindbis viruses. Additionally, simplified assays to detect IgM antibodies, which develop early after the onset of clinical illness, were developed for some of these viruses. Optimization of each assay was accomplished, and production of appropriate reagents in sufficient volume for preliminary field testing was undertaken.

Development of rapid diagnostic tests for *Bacillus anthracis* was also pursued. Significant achievements included establishment of enzyme-linked immunosorbent assay (ELISA) methods to determine antibody titers to *B. anthracis* capsule, fluorescent antibody staining of capsule, production of monoclonal antibody for *B. anthracis* vegetative cells, and characterization of 12 monoclones to *B. anthracis* spores and three to vegetative cells.

Summary:

Rapid (3-h), simple antigen and antibody detection assays developed in previous years have been adapted and perfected. Monoclonal antibodies against sandfly fever virus (Sicilian) have been produced, characterized, then optimized for use in rapid assays. Monoclonal antibodies have been acquired and employed to improve sensitivity and specificity of assays for Rift Valley fever, Crimean-Congo hemorrhagic fever, Hantaan (Korean hemorrhagic fever), and Chikungunya viruses. Validation of each assay with field-collected, epidemiologically relevant samples has continued. The inactivation of certain biohazardous viruses used as antigens in these assays has been studied. Under specific controlled conditions, both cobalt and β-propiolactone were found to inactivate Hantaan virus while retaining virus

antigenicity. Additionally, these types of assays were studied to determine the amount of antigen present in various lots of vaccine. Preliminary results indicate these assays might be useful in replacing animal inoculation tests to predict the potency of vaccines under development.

Progress:

Considering the diverse number of infectious agents which might be a threat to troops in a tropical area or as agents for a biological warfare attack, a decision was made in previous years to concentrate our efforts on a standard test - the enzyme-linked immunosorbent assay (ELISA). Additionally, our efforts initially concentrated on viral threats, especially those militarily relevant viruses where private sector and commercial research and development programs are nonexistent.

We decided to establish an ELISA for antigen and antibody detection because of the test's simplicity, speed, reliability, and sensitivity. Although recently developed, the ELISA is gaining widespread acceptance. To date, it is the most sensitive, non-isotope, antigen-detection system developed. Since the Army's rapid diagnosis extramural program has contracted with a number of companies to develop innovative new technology potentially more sensitive than the existing ELISA systems, the Rapid Diagnosis Section at USAMRIID has concentrated most of its efforts on perfecting and applying established technology.

For the antigen-detection ELISA, a standard method is employed for all viruses. A capture antibody is first bound to a solid phase, usually the bottom of a polystyrene microtiter plate. The sample is added to the plate, and, if antigen is present in sample, it is trapped to the plate by the capture antibody. After a washing step, the presence of captured antigen is determined by the addition of an enzyme-labeled detector antibody. Colorless substrate develops color if enzyme-labeled detector antibody is bound to the entrapped antigen.

The format for the IgM assay is to bind anti-human-IgM antibody to the plate. When human sera are added, a portion of the total IgM antibody is trapped to the plate. The presence of trapped, specific IgM directed against the virus is then determined by the addition of antigen, then an enzyme-labeled, anti-viral detector antibody, then substrate.

The only disadvantage of antigen-detection ELISA systems for viruses has been their lower sensitivity compared to traditional (and slower) cultivation methods. Consequently, as in previous years, much of our effort was directed at increasing ELISA sensitivity. Previous results indicated that sensitivity could be increased if monoclonal antibodies (MAB) were employed in our assays. Monoclonal antibodies are bioengineered antibodies which are pure, potent, constantly available, and can be selected for defined avidity and specificity (either very specific or broadly cross-reactive). With both Rift Valley fever and Venezuelan encephalomyelitis viruses, numerous early experiments indicated that MAB or mixtures of MAB functioned as well as or better than a number of polyclonal antisera. Mixtures of MAB to different sites on the antigen provided excellent ELISA capture and detector antibodies which increased the sensitivity of the antigen-detection ELISA when tested with various strains of virus prepared in cell culture, animals, or mosquitoes. However, not all MAB were suitable. A large bank of MAB will be needed for each agent and each individual antibody has to be evaluated in the ELISA. However, once developed, MAB will be useful in both antigen-detection and antibody-detection ELISA systems.

During 1985, much effort was concentrated on developing or obtaining from private or governmental sources MAB potentially useful in increasing sensitivity of our ELISA systems. Each antibody was evaluated for its usefulness, then grown in sufficient quantities for incorporation in rapid diagnosis kits. Some of these antibodies were developed in-house while others were produced under contract. The biological reagent support of contracts, which is frequently substantial, is also a responsibility of the intramural rapid diagnosis group. Table 1 summarizes 1985 progress made in this area. Notable progress includes initiation of testing of procured Hantaan virus (Korean hemorrhagic fever) MAB, and development and testing of over 100 MAB for sandfly fever (Sicilian strain) virus. There had been major problems developing sophisticated ELISA systems for this latter fastidious virus. The sandfly viruses (both Sicilian and Naples strains) are among the more difficult viruses to grow, purify, and prepare antigen and antibody reagents.

Both antigen-detection and IgM antibody-detection ELISA systems for sandfly fever (Sicilian) and Hantaan Viruses were optimized with epidemiologically relevant samples obtained from infected patients. After establishment of these assays, they were standardized and made available for field testing. Field testing of these assays is funded through project number 3M463750D809, work unit number 809-EA-001. Briefly, these field tests showed the assay for sandfly fever was rapid and accurate in detecting antigen and antibody during a human-use antiviral drug trial. The Hantaan assays have been transferred to People's Republic of China for use during a collaborative human-use antiviral drug protocol with Hantaan-infected patients. Preliminary results have been encouraging.

Further progress in 1985 has been made with intramural collaborative efforts to develop and characterize MAB to Crimean-Congo hemorrhagic fever virus. Characterization has begun on five MAB to Crimean-Congo hemorrhagic fever prepared under contract, and a much larger bank of MAB is being prepared through joint studies with other investigators at USAMRIID. MAB to Chagres and West Nile viruses are being developed through contracts; antigens, assays, reagents, etc., are being supplied under this work unit.

Production of large quantities of reagents for kits to be tested in the field has progressed well during 1985 (Table 1). For many antibody assays, safe, inactivated antigens must be supplied. Our previously established, standard β-propiolactone inactivation procedures did not successfully inactivate Hantaan virus. Progress was made on this problem, and higher concentrations of β-propiolactone in combination with cobalt irradiation successfully inactivated Hantaan Virus without destroying its antigenic properties.

In addition to their use for diagnosis, antigen-detection ELISA systems might be useful to detect the presence of important immunizing antigens in preparations of inactivated vaccines. To explore this, various lots of Venezuelan equine encephalomyelitis vaccines were tested for presence of antigen in an ELISA. This test utilized a MAB against a major neutralizing site on the virion to bind the virus to the ELISA plate. In general, vaccines which were potent, as judged by mouse protection tests, had the highest titer of antigen in the antigen-detection ELISA. Lots of vaccine that were poorer in immunizing mice had lower antigen titers in the ELISA. The differences, although reproducible, were not large (lowest ELISA titer 1/16 to 1/32; highest titer 1/256). Obviously, further work is required.

PRESENTATIONS:

1. **Meegan, J. M., and J. W. LeDuc.** Successful use of rapid viral diagnostic assays in lesser developed countries. Presented, National Academy of Sciences Institute of Medicine joint meeting with the Board on Science and Technology for International Development of the National Research Council. May, 1985.

2. **MacDonald, C., K. McKee, J. Huggins, J. Morrill, J. Meegan, C. J. Peters, and P. Canonico.** Ribavirin prophylaxis of sandfly fever - Sicilian infection in human volunteers. Presented, Interscience Conference on Antimicrobial Agents and Chemotherapy. September, 1985.

3. **Tammariello, R., M. Faran, J. Meegan, and C. Bailey.** Application of ELISA for detection of Rift Valley fever virus in mosquitoes. Presented, Annual Meeting of the American Society of Tropical Medicine and Hygiene. December, 1984.

4. **Nuzum, E.** Development of a rapid viral antigen detection ELISA for respiratory viruses. Presented, Staff Conference, Brooks AFB. August, 1985.

5. **Meegan, J., L. Laughlin, and N. Birgis.** Enzyme immunoassay detection of IgM and IgG antibodies to Rift Valley fever in cerebrospinal fluid. Presented, 6th International Congress of Virology. September, 1984.

6. **Meegan, J. M.** Evaluation of diagnostic kits produced by phase I contracts under identification and diagnostic system biological agent, rapid, field (IDSBARF) program. Presented, IDSBARF Source Selection Board. July, 1985.

PUBLICATIONS: None

TABLE 1. Progress in developing monoclonal antibodies and their use to increase sensitivity and reproducibility of rapid antigen or antibody detection assays.

Monoclonal to[a]	Developed/ procured	Characterized	Optimized for ELISA	Tested with field samples	Produced in large quantities
RVF	Yes (DoD) (1982-84)	Yes (1982-85)	Yes (1984)	Yes (1984-85)	Yes (1984-85)
VEE	Yes (CDC) (1984)	Yes (1984)	Yes (1984)	Yes (1984)	Yes (1984-85)
CCHF	In progress	In progress	In progress	----	----
SFS	Yes (1985)	In progress	In progress	Yes (1985)	In progress
HTN	Yes (CDC) (1984)	Yes (1984)	Yes (1985)	In progress	Yes (1984)
CHIX	In progress (Virol Div)	In progress (Virol Div)	In progress	In progress	----
CHG	In progress	----	----	----	----
WN	In progress	----	----	----	----

[a]RVF= Rift Valley fever virus
VEE= Venezuelan equine encephalomyelitis virus
CCHF= Congo-Crimean hemorrhagic fever virus
SFS= Sandfly fever virus (Sicilian)
HTN= Hantaan virus
CHIK= Chikungunya virus
CHG= Chagres virus
WN= West Nile virus

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 302670	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763A	3M263763D807	AI	018
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Development of Vaccines Against Botulism

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0620 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10 01	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	1.1	153
86		

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Johnson, A J
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7211
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Crumrine, M H
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Siegel, L S

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Microbial Toxins; (U) Vaccines; (U) Therapy; (U) Toxoids; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Evaluation and testing of toxiods and antitoxins for protection against botulinal neurotoxins. These neurotoxins are considered to have significant biological warfare potential and our at-risk forces should be immunized against them.

24. (U) Obtain botulinum toxin serotypes A-G in partially or highly purified state. Evaluate toxiods prepared from such materials for protection of personnel against botulinum toxin poisoning. Simultaneously evaluate antitoxins produced against botulinal toxiods as suitable prophylactic or therapeutic agents for botulinal toxin poisoning.

25. (U) 8410-8509-Botulinum type A toxoid preparations, collected during varying stages of toxin purification and toxoided by formalin treatment, were nontoxic when tested in mice and guinea pigs. Their immunogenic strengths were compared to a known immunogenic monovalent botulism type A toxoid prepared by the Michigan Department of Public Health (MDPH). As the amount of specific toxin protein was increased in the toxoid, resulting titers in immunized guinea pigs showed a significant increase. An RFP was prepared and issued for the production of a hexavalent (ABCDEF) botulinal toxoid. A second RFP was developed and issued for a research effort to develop a technological base to evaluate the feasibility of adding type G toxoid to a multivalent botulinal toxoid.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AI-018: Exploratory Development of Vaccines Against Botulism

PRINCIPAL INVESTIGATOR: Anthony J. Johnson, LTC(P)

ASSOCIATE INVESTIGATORS: M. H. Crumrine, Ph.D. MAJ
L. S. Siegel, Ph.D.
A. D. Johnson-Winegar, Ph.D.

Background:

There are seven immunologically distinct neurotoxins (types A-G) produced by the heterogeneous group of bacteria given the genus species designation *Clostridium botulinum*. Types A, B, E, and F have been identified in cases of human disease. Types C, D, and G have the potential of producing toxic effects in man, but there have been few well-documented cases reported. Toxoids (chemically inactivated but immunogenic toxins) for each serotype are used to induce immunity to these toxins. The botulinal toxoid used for immunization of humans was produced by the Michigan Department of Public Health (MDPH) under contract to the Army in the late 1960's by treating types A, B, C, D, and E toxin with formaldehyde. This toxoid produces sustained, measurable antibody levels, but only after a series of four injections over a period of one year. Mild side effects, including tenderness, redness, swelling, and induration at the site of injection, are frequent. An improved toxoid prepared from more highly purified neurotoxins is required. It should include, in addition to the current pentavalent toxoid, types F and G.

Summary:

Botulinum type A toxoid preparations, collected during varying stages of toxin purification and toxoided by formalin treatment, were nontoxic in mice and guinea pigs. Their immunogenic strengths were compared to a known immunogenic monovalent botulism type A toxoid prepared by the MDPH. As the amount of specific toxin protein was increased in the toxoid, resulting titers in immunized guinea pigs showed a significant increase. An Request For Proposal (RFP) was prepared and issued for the production of a hexavalent (ABCDEF) botulinal toxoid. A second RFP was developed and issued for a research effort to develop a technological base to evaluate the feasibility of adding type G toxoid to a multivalent botulinal toxoid.

Progress:

The effect of purification of the toxin on the efficacy of the corresponding toxoid has been examined by Drs. Siegel and Johnson-Winegar. Toxoids were prepared from type A toxin at three steps of the purification procedure: the crude culture extracts, the toxin peak from an ion exchange column (citrate peak, 40-50% neurotoxin), and the neurotoxin peak from another column chromatography step (phosphate peak, 85-90% pure). All preparations were incubated at 35°C in the presence of 0.6% formalin for 25 days. After being tested for residual toxicity and found to be nontoxic, preparations were adsorbed to aluminum hydroxide and used to immunize guinea pigs. For comparative purposes, a monovalent, type A toxoid prepared by the

Michigan Department of Public Health (MDPH) under contract to the U.S. Army was also used to immunize guinea pigs. All preparations protected the animals from an i.p. challenge, 28 days later, with 10^5 LD_{50} of type A botulinum toxin. To measure the antibody response to the toxoids, a serum sample was obtained from each guinea pig two days prior to challenge. The serum from each of 15 animals in any group was assayed individually for antibody (by using a toxin neutralization test in mice with World Health Orgainization monovalent A antitoxin as a standard). Results are shown in Table 1.

These studies demonstrated the variation in antibody response among animals receiving the same toxoid. This wide range of values would have been masked if the sera for animals in each group had been pooled for assay rather than assayed individually. The antibody response to the MDPH toxoid and to the first two products were not statistically different. The second toxoid (citrate, 40 to 50% pure) contained about twice as much neurotoxoid as did the crude material, but the antibody response was not statistically greater. Thus, purification to this point is of questionable value. However, we demonstrated that toxoid prepared from the more highly purified toxin did indeed elicit a higher antibody response, when compared on a protein basis with the other products. These results were confirmed in follow-up studies. This is an obvious product improvement. Also, this approach in acquiring an improved immunogen (toxoid) allows the product to remain within the acceptable FDA-guidelines. An anticipated bonus would be that injection of less foreign protein may also elicit fewer side reactions.

The accepted standard for assay of the antibody response to botulinum toxoids is the neutralization test. It is a bioassay, requiring 20 mice to assay one serum sample. It is also time-consuming, since a four-day observation period for mouse survival is required. Therefore, the serum samples were also assayed by an ELISA system. We are developing a data base to correlate ELISA values with neutralization titers, so that the ELISA may replace the neutralization test.

An RFP, Solicitation #DAMD 17-85-R-0079, was issued for the development and production of 40,000 doses of a blended product that would contain type A, B, C, D, E, and F toxoids. A second RFP, Solicitation #DAMD-17-85-R-0080, was issued for developmental studies to evaluate the feasibility of producing type G botulinal toxoid, and including this toxoid in a final heptavalent (ABCDEFG) product. An option to produce eight million doses of either a hexavalent or a heptavalent toxoid was included in the initial solicitation. Both RFPs have been published and a Source Selection Board is evaluating proposals from prospective vendors. Selection of a vendor and subsequent contract award should be made in FY 86.

PRESENTATIONS:

1. Siegel, L. S. Toxins of *Clostridium botulinum*. Presented at the Bacterial Vaccine Symposium, sponsored by and convened at the National Institutes of Health, 1984.

2. Siegel, L. S. Effect of purification of type A botulinum toxin on the efficacy of the corresponding toxoid. Presented at the Interagency Botulism Research Coordinating Committee Meeting, 1984.

3. Siegel, L. S. Development of type A botulinum toxoids. Presented at the USAMRIID Professional Staff Conference, 1985.

4. **Siegel, L. S., and A. Johnson-Winegar.** Effect of purification of type A botulinum toxin on the efficacy of the corresponding toxoid. Presented at the Annual Meeting of the American Society for Microbiology, Las Vegas, NV, 1985.

5. **Johnson-Winegar, A., and L. S. Siegel.** Analysis of the humoral immune response in guinea pigs vaccinated with toxoids of type A *Clostridium botulinum*. Presented at the Annual Meeting of the American Society for Microbiology, Las Vegas, NV, 1985.

6. **Crumrine, M. H.** Experimental therapy for botulism with equine $F(ab)_2$ antitoxin. Presented at the Interagency Botulism Research Coordinating Committee Meeting, 1984.

PUBLICATIONS:

1. **Siegel, L. S., A. D. Johnson-Winegar, J. J. Schmidt, L. A. Smith, M. H. Crumrine, and J. L. Middlebrook.** 1984. Toxins of *Clostridium botulinum*. Proceedings of the Bacterial Vaccine Symposium (In Press).

TABLE 1
Neutralization antibody response to Type A botulinal toxiods

		TOXOIDS			
		MDPH	Extracts	Citrate	Phosphate
Neutralization	Mean	2.13[a]	1.90	2.58	15.2
Titers:					
(IU/ml)	Low	0.36	0.02	0.28	1.0
	High	7.20	5.10	5.10	32.5

[a]Titers are expressed in International Units per ml.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 302668	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763D	3M263763D807	AG	019
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) (U) Immunotherapy of Hemorrhagic Fever Viruses

12. SUBJECT AREAS
0613 Microbiology; 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE — EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE — d. AMOUNT
e. KIND OF AWARD — f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	0.4	215
86	0.0	235

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Disease Assessment Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Jahrling, P B
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7244
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available)
MILITARY/CIVILIAN APPLICATION M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Lassa Virus; (U) Viral Diseases; (U) Lab Animals; (U) Primates; (U) Guinea Pigs; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To select, acquire, and test immune plasma and globulin fractions for protective efficacy and safety in prophylaxis and therapy of hemorrhagic fever virus infections which pose special problems for U.S. Forces sent to those areas where these diseases are endemic.

24. (U) Specific immune plasma is obtained by plasmapheresis from convalescent patients after naturally occurring infections with Lassa virus, Argentine hemorrhagic fever virus (Junin), and Ebola virus. Plasma units are tested by current blood bank procedures and for presence of protective (neutralizing) antibodies. Criteria are established for optimal therapeutic administration of the final products. Alternate strategies for acquiring high titered antibody are developed and tested.

25. (U) 8410 - 8509 - The pool of Lassa fever (LV) convalescent patients in Liberia has been expanded to include 77 with modest to high titers of neutralizing antibody (LNI). Of 344 plasma units received, 215 (77%) have detectable LNI, and 151 ([illegible].1%) contain sufficient LNI titers to confer protection without further processing. From Sierra Leone, 191 have been received: 34 can be used directly, 109 require processing, and 49 contain insufficient LNI. All units were tested for HTLV-III antibody by Abbott ELISA; 2 donors were thus excluded. Confirmatory Western blots are in progress; 3 more donors were HBsA9 positive. Processing of human plasma by chromatographic methods resulted in similar yields of effective LNI. Pending results of HTLV recovery, 1 process will be selected for preparation of a pilot lot of IgG for extended efficacy testing.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AG-019: Immunotherapy of Hemorrhagic Fever Viruses

PRINCIPAL INVESTIGATOR: P. B. Jahrling, Ph.D.

Background:

Lassa fever is a viral disease of considerable public health importance in regions of West Africa, particularly Liberia, Sierra Leone, and Nigeria, where several thousand cases are believed to occur annually (1). While serological data suggest that subclinical cases may occur, the case-fatality ratios among hospitalized cases are still high, variously estimated at 14 to 22% in Sierra Leone and 13 to 14% in Liberia. To increase survival rates, passive immunization of acutely ill patients is employed frequently (2). One of many problems in evaluating plasma efficacy is the variable quality of the plasma infused. This study was designed to identify Lassa fever convalescent patients to be recruited as plasma donors. Through this process, a pool of optimal donors was identified, and guidelines for identifying new donors were established. The availability of high-titer plasma has now facilitated the formal testing of passive immunization for treatment of Lassa fever.

Summary:

The pool of Lassa fever (LV) convalescent patients in Liberia has been expanded to include 77 modest to high titers of neutralizing antibody (LNI). Of 344 plasma units received, 215 (77%) have detectable LNI, and 151 (54.1%) contain sufficient LNI titers to confer protection without further processing. From Sierra Leone, 191 have been received: 34 can be used directly, 109 require processing, and 49 contain insufficient LNI. All units were tested for HTLV-III antibody by Abbott ELISA; two donors were thus excluded. Confirmatory western blots are in progress; three more donors were HBsA9 positive (1). Processing of human plasma by chromatographic methods resulted in similar yields of effective LNI. Pending results of HTLV recovery, one process will be selected for preparation of a pilot lot of IgG for extended efficacy testing.

Progress:

Acquisition and testing of human Lassa-convalescent plasma Plasma-collection efforts have continued in collaboration with Dr. John Frame and colleagues, under contract, in Liberia. The quality of plasma, with respect to indirect fluorescent antibody (IFA) and neutralizing antibody titers determined at USAMRIID, continues to improve, as prospective donors with LNI titers known to be high are identified and recruited to donate multiple units of plasma. Of 135 plasma units received during the reporting period, 80 have been tested for IFA and LNI (Table 1). Seventy-seven of 80 (96%) had IFA to Lassa, and 72 of 80 (90%) had at least some neutralizing antibody (LNI > 0.3). Fifty of 80 (63%) had LNI > 1.5, which is considered sufficient to confer protection without further processing. From Sierra Leone, no further plasma units were received from Dr. Joe McCormick. The total inventory of Lassa-immune plasma is listed in Table 2.

In our continuing effort to identify new prospective plasma donors, sera from 543 patients hospitalized in Zor-Zor, Liberia, with febrile illnesses compatible with Lassa fever were tested for viral infectivity. Sixty-six viral isolates were obtained and identified as Lassa fever by neutralization testing. Twenty-nine additional patients were identified as Lassa-convalescent by seroconversion. Additional testing by ELISA and isolation attempts from an additional backlog of over 1500 sera recently were resumed after an unscheduled renovation of the Institute's Lassa suite and a 1-year sabbatical leave of the principal investigator.

Testing of immune plasma units for adventitious agents Samples of 344 plasma units were submitted to Dr. Redfield at WRAIR for hepatitis B surface-antigen testing and antibodies to HTLV-III virus (AIDS) by Abbott ELISA. Plasma from 8 of 77 donors was excluded on the basis of hepatitis reactivity, and two for low HTLV-III titers. Confirmatory western blots are promised. In the future, these and additional plasma samples will be submitted to an independent, certified laboratory for this and related testing prior to their inclusion in plasma pools scheduled for fractionation (see below).

Combination of immune globulin with ribavirin The protective efficacies of various preparations of human immune globulins were enhanced by combination with suboptimal (and nontoxic) concentrations of ribavirin (Table 3). Immunoglobulins prepared by the QAE and CM chromatographic methods were known to be fully protective when administered in doses of 3 ml/kg or more; lower doses were suboptimal. Ribavirin alone (20 mg/kg) was also suboptimal. However, combination of ribavirin plus either QAE or CM globulins at 1.5 ml/kg significantly enhanced protection. In contrast, the combination of F(ab')2 with ribavirin did not enhance efficacy over ribavirin alone. These results were reflected in the viremia patterns in the treated guinea pigs. Thus it is reasonable to predict that protective efficacies of immune globulins for human Lassa fever patients might be improved by combination with ribavirin, similar to the observation of enhanced protection with whole plasma plus ribavirin when treatment was delayed in cynomolgus monkeys (4).

Protection of monkeys and guinea pigs with immune plasma and globulin fractions (ALF#2) The preparation and preliminary testing of immune globulins by two different procedures (QAE and CM-chromatography), in collaboration with Dr. Richard Condie, was reported in FY84. In a continuation study, the protective efficacies of these products were determined in cynomolgus monkeys and guinea pigs infected with Liberian Lassa virus strains. Both QAE and CM globulin protected monkeys and totally inhibited viremia when administered in high dose, (6 ml/kg i.v., days 0, 3, and 6) (Table 4). Unfractionated globulin was marginally more protective, but both QAE and CM material was significantly more protective than unfractionated plasma.

Comparison of Cohn-ethanol fractionated globulin with QAE and CM globulins A third human plasma pool (ALF#3) was fractionated and five different products were compared for LNI and protective efficacy in guinea pigs infected with our reference Sierra Leone Lassa virus (strain Josiah) and a Liberian strain (Z-132). Cohn-ethanol fraction II was prepared since this material is believed to be relatively safe with respect to transmission of adventitious agents including hepatitis and AIDS viruses. Some of this material was treated with pepsin to disaggregate globulin clumps, thus permitting intravenous administration (5). Unfractionated plasma was marginally effective at 3 ml/kg and poor against Josiah at 1.5 ml/kg (Table 5). F(ab')2 fragments had significantly reduced LNI titers relative to the plasma pool, and this product was totally devoid of protective activity. As with the ALF#2 preparations, CM-fractionated globulin had marginally higher titers and was more protective than QAE-globulin against both challenge virus strains. Both the Cohn-ethanol fraction and the pepsin-disaggregated material were highly protective at 3 ml/kg; testing of lower doses is in progress. These results from guinea pigs will be confirmed by testing in cynomolgus monkeys.

The choice of a fractionation procedure for preparing immune globulin for intravenous administration to human Lassa fever patients will be based on a combination of the protective efficacy results, comparative immune globulin yields, and an assessment of the comparative risks of contamination of the final products with adventitious agents including hepatitis B and HTLV-III viruses.

PRESENTATIONS:

1. **Jahrling, P. B., C. J. Peters, G. A. Eddy, and J. I. Maiztegui.** Development of effective viral-specific treatment strategies for arenaviral hemorrhagic fevers. Presented, XI International Congress for Tropical Medicine and Hygiene, Calgary, Canada. Sept 84.

PUBLICATIONS:

1. **Jahrling, P. B., J. D. Frame, J. B. Rhoderick, and M. H. Monson.** 1985. Endemic Lassa fever in Liberia. IV. Selection of optimally effective plasma for treatment by passive immunization. *Trans. R. Soc. Trop. Med. Hyg.* 79:380-384.

2. **Jahrling, P. B., and C. J. Peters.** 1985. Arenaviruses, pp. 171-189. *In* E. H. Lennette (ed.), Laboratory diagnosis of viral infections, Chapter 11. Marcel Dekker, Inc., NY.

3. **Jahrling, P. B., B. S. Niklasson, and J. B. McCormick.** 1985. Early diagnosis of human Lassa fever by ELISA detection of antigen and antibody. *Lancet* 1:250-252.

LITERATURE CITED

1. **McCormick, J. B., and K. M. Johnson.** 1984. Viral hemorrhagic fevers, pp. 676-701. *In* Warren and Mahmoud (ed.), Tropical and geographic medicine. McGraw-Hill, NY.

2. **Monath, T. P., and J. Casals.** 1975. Diagnosis of Lassa fever and the isolation and management of patients. *Bull. W.H.O.* 52:707-715.

3. **Clayton, A. J.** 1977. Lassa immune serum. *Bull. W.H.O.* 55:435-439.

4. **Jahrling, P. B., C. J. Peters, and E. L. Stephen.** 1984. Enhanced treatment of Lassa fever by immune plasma combined with ribavirin in cynomolgus monkeys. *J. Infect. Dis.* 149:420-427.

5. **Walsh, J. J.** 1974. Studies on the purification of normal immunoglobulin for intravenous use. *Dev. Biol. Stand.* 27:31-36.

Table 1. Inventory of plasma units collected in Liberia for use in treatment of Lassa fever

Date received	No. units	IFA≥10 No. (%)	LNI ≥0.3	LNI≥1.5
Oct 80-Mar 83	131	99 (75.5)	79 60.3)	48 (36.6)
Aug 83	18	18 (100.0)	18 (100.0)	11 (61.1)
Nov 83	26	26 (100.0)	24 (92.3)	13 (50.0)
May 84	24	24 (100.0)	22 (91.6)	19 (79.1)
Oct 84	34	32 (94.1)	29 (85.3)	21 (61.7)
Jan 85	16	16 (100.0)	14 (87.5)	9 (56.2)
May 85	30	30 (100.0)	29 (96.6)	20 (66.7)
	279[a]	245 (87.8)	215 (77.0)	141 (50.5)

[a]Plus 65 units awaiting testing (Aug 85).

Table 2. Inventory of African plasma units collected for treatment of Lassa fever

		IFA (%)		LNI (%)		
Origin	Units Received	Tested	IFA≥10	Tested	≥0.3	≥1.5
Liberia	344	279	245 (87.8)	279	215 (77.0)	141 (50.5)
Sierra Leone	191	187	131 (70.0)	170	109 (64.1)	34 (20.0)

Summary: Aug 85

Table 3. Protection of cynomolgus monkeys against Lassa virus (Liberian Strain Z-158) by human immune plasma or plasma concentrates (ALF#2)

Treatment	LNI	Dose (ml/kg)[a]	Dead/Total	Days of Death	Viremia-$\log_{10}$ Day 7	PFU/ml on Day 14
Plasma	1.6	6	3/4	21,24,29	<	4.6
		3	2/2	16,23	2.3	4.7
QAE	3.0	6	0/3	---	<	<
CM	3.5	6	0/2	---	<	<
None	---	---	4/4	11,13,13,15	3.5	4.0

[a]Days 0,3,6; inoculated i.v.

Table 4. Protection of guinea pigs against Lassa virus (Liberian strain Z-132 by human immune plasma or plasma concentrates (ALF#2)

Treatment	LNI	Dose (ml/kg)[a]	Dead/5	MTD[b]	Viremia-$\log_{10}$ Day 7	PFU/ml Day 14
Plasma	1.6	6	3	28.0	<	2.0
		3	5	18.2	2.2	4.3
QAE	3.0	6	0	---	<	<
		3	1	(29)	<	<
		1	5	21.2	<	3.0
CM	3.5	6	0	---	<	<
		3	0	---	<	3.1
None	---	---	5	18.4	3.3	3.6

[a]Days 0,3,6; inoculated i.p.

[b]Mean time to death.

Table 5. Protective efficacy of human immune globulin (ALF#3) preparations for guinea pigs inoculated with Lassa virus strains

Treatment:	Challenge Virus				Results of Challenge	
Human ALF#3[a]	Strain[a]	IFAT	LNI	Dose (ml/kg)[b]	% Protected (n=5)	MTD[c]
Unfractionated	JOS	640	1.6	3	40	19.6
Plasma				1.5	20	19.0
	Z132	640	1.9	3	40	21.0
$F(ab')_2$	JOS	1280	0.6	3	0	19.5
				1.5	0	20.2
	Z132	1280	1.0	3	0	21.0
				1.5	0	20.0
Intact IgG (QAE)	JOS	1280	2.6	3	80	23
				1.5	20	20.0
	Z132	1280	2.8	3	80	20
				1.5	60	20.5
Intact IgG (CM)	JOS	1280	2.8	3	60	17.5
				1.5	60	22.0
	Z132	1280	3.2	3	100	--
				1.5	80	26
Cohn Fraction II	JOS	1280	2.0	3	80	19
	Z132	1280	2.6	3	100	--
Cohn (Pepsin-disaggregated)	JOS	1280	2.1	3	100	--
	Z132		3.4	3	100	--
None	JOS	--	--	--	0	21.6
	Z132	--	--	--	0	20.5

[a]JOS=Josiah Strain, Sierra Leone. Z132=Human Lassa Virus, Liberia.

[b]Immune globulin administered i.p. on days 0, 3, and 6.

[c]Mean time to death.

Table 6. Efficacy of human immune globulin fractions, ribavirin, or combined therapy in treatment of strain 13 guinea pigs infected with Lassa virus, strain Josiah

Treatment					Viremia (log_{10}PFU/ml)		
Human ALF#3[a] days 0,3,6	Globulin Dose ml/kg	Ribavirin[b] 20 mg/kg d0-10	Dead/Total	MTD[c]	d7	d14	d20
---	---	+	4/5	21.0	<	2.28	3.02
QAE	1.5	---	4/5	20.0	<	2.83	3.92
QAE	1.5	+	0/5	---	<	1.32	1.64
CM	1.5	---	2/5	22.0	<	1.84	1.55
CM	1.5	+	0/5	---	<	1.02	1.21
$F(ab^1)2$	1.5	---	5/5	19.4	2.84	3.54	2.82
$F(ab^1)2$	1.5	+	5/5	22.0	<	2.10	2.95
---	---	---	5/5	18.2	3.34	3.83	(dead)

[a]LNI versus Lassa strain Josiah: QAE (2.8); CM (3.3); $F(ab^1)2$ (1.1).

[b]Ribavirin dose divided by 2 and inoculated at 12-h intervals i.p.

[c]Mean time to death.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
		DA 302660	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763	3M263763D807	AL	020
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Development of Vaccines, Therapeutic Agents and Immunomodulators Against Aerosols

12. SUBJECT AREAS
0605 Clinical Medicine; 1503 DEFENSE

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE | EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE | d. AMOUNT
e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	3.9	793
86		

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Airborne Diseases Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Stephenson, E H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Anderson, A O
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Scott, G H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Mice; (U) Vaccines; (U) Microorganisms (U) Aerosols; (U)Lab. Animals; (U)Hamsters; (U)Guinea Pig

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Prophylactic vaccines, therapeutic agents, and immunomodulators developed to protect US troops against potential BW agents must be effective when the microorganism or toxin is presented in an aerosol. The objective is to determine the safety, efficacy, and dose response of prophylactics, therapeutics, and immunomodulators against an airborne challenge. Emphasis is on how effective products induce protection and why ineffective ones fail.

24. (U) Define and quantify the molecular aspects of clinical, pathological, and immunological changes that occur in vaccinated or treated animals when exposed to potential BW agents in aerosols. Apply advanced methodology to determine how a prophylactic or therapeutic induced protection; or, conversely to analyze why a product failed to instill protection.

25. (U) 8410-8509 - Attenuated, non-encapsulated Sterne strain of *Bacillus anthracis* consistently induced greater protection in guinea pigs against an aerosol challenge, as compared to protective antigen (PA) products. At 39 weeks after vaccination, 50% of Sterne-vaccinated animals survived; only 30% of the PA-vaccinated guinea pigs survived. Three attenuated strains of Rift Valley fever virus did not induce protection in more than 75% of the mice or hamsters given a modest airborne challenge, which approximated the results obtained with formaldehyde-inactivated RVF virus vaccine. Candid #1 Junin virus vaccine imparted protection in guinea pigs against a significant virus challenge in aerosol. Animals receiving more than 30 PFU of the Candid #1 virus lived, did not yield virus from tissues through 60 days after challenge, did not develop any detectable pathologic lesions, and exhibited neutralizing antibodies by day 60. The only animals that died were those that received less than 1 PFU of vaccine virus.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AL-020: Exploratory Development of Vaccines, Therapeutic Agents and Immunomodulators Against Aerosols

PRINCIPAL INVESTIGATOR: E. H. Stephenson, D.V.M., Ph.D., COL, VC

ASSOCIATE INVESTIGATORS: A. O. Anderson, M.D., LTC, MC
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G. H. Scott, Ph.D.

Background:

Any agent to be employed as a biological weapon against US forces must cause infection or intoxication via the respiratory tract. Investigators are assigned to evaluate vaccines, therapeutic agents, and immunomodulators that have been developed for candidate BW agents. The studies are aimed at identification of efficacious prophylactic and therapeutic regimens which will protect deployed US troops and at-risk laboratory personnel.

Summary:

Major accomplishments within the work unit were in the following areas: 1) on a basis of percent survival and median time to death, neither any combination of anthrax toxins (PA, EF, or LF), nor the current human vaccine (MDPH) were as effective in protecting guinea pigs against aerosol challenge as the attenuated, non-encapsulated, Sterne Strain of *Bacillus anthracis*. The superiority of the Sterne vaccine was even more pronounced when protection was studied for 39 weeks after vaccination. Fifty percent of spore-vaccinated guinea pigs survived virulent challenge at this time in contrast to 30% of protective antigen (PA)-vaccinated animals. All of the guinea pigs that received a booster dose of Sterne Vaccine 12 weeks after initial vaccination survived virulent challenge, while only 40% of PA-boosted animals survived. The dose of Sterne spores required for complete protection 21 days after vaccination was 2.5×10^6 spores. 2) Three attenuated strains of Rift Valley fever (RVF) virus (Caplan, Moussa, Smithburn) did not induce protection in more than 75% of the mice or hamsters given a modest airborne challenge, which approximated the results obtained with formaldehyde-inactivated RVF virus vaccine. 3) The A/J mouse is more sensitive to phase I *Coxiella burnetii* infections than is the C57BL/6J mouse, as measured by the number of *C. burnetii* required to kill 50% of the animals and by the number of *C. burnetii* required to induce suppression of splenic lymphocyte responses. The lymphocyte proliferation assay was a more sensitive indicator of the difference between the inbred strains. Suppression of concanavalin A (ConA) responsiveness in the A/J strain was induced with only one infectious rickettsia, whereas a dose of $10^{6.7}$ rickettsiae was required to cause immunosuppression in the more resistant C57BL/6J strain.

Progress:

Anthrax The persistence of immunity to *B. anthracis* was studied by immunizing five groups of approximately 150 guinea pigs each with 3 i.m. injections of either 0.5 ml of commercial, adsorbed, partially purified PA vaccine (MDPH) or three vaccinations of Sterne strain spores (Anvax) at 2-week intervals. The Sterne vaccine contained 2 $\times 10^6$, 4×10^6 spores in 0.2, 0.4, or 0.5 ml, respectively. At 12 weeks, 48 guinea pigs in each of the vaccine groups were given homologous booster-doses of Sterne or MDPH vaccines. Approximately 100 control animals were vaccinated with sterile saline solution.

At 2, 4, 8, 16, and 39 weeks after the last vaccination, six guinea pigs from each group were bled for serology and killed with an overdose of halothane. A bronchoalveolar washing (BAW) was obtained by flushing the lungs and trachea with 3.0 ml of saline solution. The material obtained was frozen pending concentration and serology. Serum antibody titers were determined by Dr. Ezzell of Bacteriology Division using the ELISA test with PA antigen. Ten animals from each treatment group were then challenged by aerosol with about 10 LD_{50} of virulent V-1-B strain of *B. anthracis*. A somewhat similar experiment, with shorter times and no boosters, has been reported by Jemski (2). The data indicate that the Sterne vaccine provides better protection against aerosol challenge than does MDPH (Table 1). Booster vaccinations were effective only for a relatively short period in the case of the MDPH. The data also show that serum antibody titers directed against PA antigen do not correlate with protection against virulent challenge. However, titers were obtained in a test that used a protein-A conjugate. Therefore, it is possible that titers of antibody of some class other than IgG may be better predictors of immunity. The titers of the antibody contained in BAW suspensions have not yet been determined. Thirty nine-week challenge experiments are currently in progress.

The shortcomings of the MDPH vaccine have led to further studies of the efficacy of Sterne, and to a consideration of the possible use of combinations of the two vaccines. The latter has been studied by vaccinating guinea pigs according to the following protocol:

1. Sterne followed at 2 weeks by another Sterne injection.
2. Sterne followed at 2 weeks by MDPH
3. MDPH followed at 2 weeks by another MDPH injection.
4. MDPH followed at 2 weeks by Sterne
5. Saline followed at 2 weeks by saline.

All animals were challenged with 10 LD_{50} of virulent V-1-B strain 6 weeks later, both by aerosol and by i.m. injection. No differences in protection between vaccinated groups were noted. All vaccinated animals survived challenge. This observation suggests that insufficient time elapsed between vaccination and challenge for differences to become apperent.

Studies of the Sterne strain preparation were begun with a dose-response study. Five groups of 12 guinea pigs each were immunized i.m. with 2.5×10^2 to 2.5×10^6 organisms. A sixth group was vaccinated with saline solution.

Four weeks later all animals were challenged by aerosol with $10_6 LD_{50}$ of the V-1-B strain of *B. anthracis*. Only the highest aerosol dose, 2.5×10^6, was fully effective in providing protection (Table 2). It is possible, of course, that a 2- or 3-dose regimen would have provided better protection. On the other hand, if Sterne replicates in the host, it seems unlikely that any advantage would follow from multiple vaccinations. The pattern of replication is currently under study.

In a previous report (3) we described some experiments done in collaboration with Dr. Welkos, Bacteriology Division, to determine the suitability of selected strains of mice as models for respiratory anthrax studies. The principal finding was that the A/J mouse could be used, but the LD_{50} was about 100 times that for guinea pigs. This study was extended to determine whether the model would be useful for immunological experiments. The Sterne strain could not be used for this purpose because it is lethal for the A/J mouse when injected in doses as low as 10^3. Four groups of 10 A/J mice each were immunized by i.m. injection of 0.02 to 20.0 µg of MDPH protein. A fifth group was injected with saline as the control. Immunizations were repeated at 2 and 4 weeks. Twenty one days later, all mice were challenged by aerosol with 10 LD_{50} of the V-1-B strain. Protection was afforded only by the highest dose of MDPH (50% survival, Table 3). This indicates that, for this vaccine, the mouse is not a suitable model. The finding also suggests that the mouse does not recognize the same antigens as the guinea pig or does not respond as well, since the guinea pig would have been almost totally protected against a 21-day challenge by the same immunization regimen.

A test of the hypothesis that calcium would "potentiate" the virulence of V-1-B strain was made in collaboration with Dr. Welkos. Two strains of mice were used: A/J and DBA/2J. In each, the response to the V-1-B strain containing 3% added $CaCl_2$ was compared to the response observed in the absence of added calcium. Calcium did not enhance the virulence of the culture.

Rift Valley fever (RVF) virus Three attenuated strains of RVF virus were evaluated as potential candidates for vaccines: Caplan (mutagen-derived), Smithburn strain (originally neurotropic, mouse brain-derived) and Moussa strain (originally non-neurotropic, plaque-derived). Each attenuated strain was compared to the formaldehyde-inactivated RVF virus vaccine in C3H/HeN mice. Vaccinated mice were challenged 21 days after vaccination by aerosol exposure to virulent ZH501 strain RVF virus.

Caplan strain-vaccinated mice given an inhaled challenge of 20 LD_{50} died of liver necrosis four days after exposure, the same as unvaccinated mice. None of the mice that received 10^6 PFU or less of the Caplan strain developed detectable serum neutralizing antibody titers to ZH501 strain.

Of 34 mice vaccinated with the Smithburn strain and evaluated for humoral antibodies 21 days after vaccination, 19 exhibited neutralizing antibodies to ZH501 strain. All mice without antibodies died after exposure; 21% of those with antibodies survived, compared to survival rate of 41% among mice given the inactivated vaccine. Survival data at 23 days after challenge were analyzed with the Cox Proportional Hazard Model. Results from the inactivated vaccine group were assigned a relative risk of 1.00. Relative risk for animals vaccinated with the Smithburn strain was 1.46, which was not statistically significant. Animals given attenuated virus but not evidencing antibody had a relative risk of 2.62, while data from unvaccinated control animals were calculated at 5.29; both group risk values were significantly different ($P > 0.005$).

The Moussa strain, like the Smithburn strain, did not induce neutralizing antibody production to the virulent virus in all recipients. Only 16 of 35 mice were found to have detectable neutralizing antibodies. Of those with antibody, 44% survived the aerosol challenge. One of 19 mice without antibody did not die after exposure. Based on the Cox Proportional Hazard Model, the relative risk for mice with antibody was 0.45, which approaches statistical significance. As expected, mice without antibody had an increased risk of 3.6.

Two death patterns were observed among the mice given the different immunogens. One occurred between days 4 and 7 after challenge. Death was due to hemorrhagic necrosis of the liver with some accompanying lymphoid necrosis. Unvaccinated mice and mice without neutralizing antibody following vaccination typically exhibit these alterations. The second death pattern occurred from day 8 to 14. The principal cause of death was necrotizing encephalitis, often with lesions extending to the brain stem. These were the observations routinely made for mice with antibodies, whether the immunogen was attenuated or inactivated virus.

Coxiella burnetii. Previous evaluations indicated that inbred A/J mice are relatively sensitive to lethal *C. burnetii*, while C57BL/6J mice are resistant. As an extension, the effects of *C. burnetii* infection in the two strains as a function of infecting doses were compared. Induction of gross pathological responses (splenomegaly and hepatomegaly) and the modulation of phase I, phase II, and LPS antibodies were studied.

Mice of each strain were injected i.p. with varying doses of the 9 Mile isolate of *C. burnetii*, phase I. Injected mice were observed daily for 30 days. Surviving mice were bled for antibody determinations, then necropsied and the liver and spleens examined for gross necrosis and weighed. Single cell suspensions of three spleens from each group were prepared and pooled, then lymphocyte proliferation assays were conducted by a modification of a microculture assay procedure. Cells were stimulated in vitro with ConA or killed phase I cells (WCI), the recall antigen.

All A/J mice injected with $10^{7.7}$ or more rickettsiae died. No A/J mice died following doses of $10^{6.7}$ or lower. Only 89% of the C57BL/6J mice died at the highest dose administered ($10^{10.7}$ rickettsiae). As determined by the Moving Average Method, the LD_{50} for A/J mice ($10^{7.1}$) was about 1000 times less than that for C57BL/6J mice ($10^{9.9}$ rickettsiae).

By comparison with spleens from saline-injected mice, a significant (P >0.05) increase in spleen mass was observed for both strains of mice inoculated with five or more viable rickettsiae. The degree of splenomegaly was essentially proportional to the dose received, but was more enhanced in the resistant C57BL/6J mice than in sensitive A/J mice. Gross liver necrosis was observed in 50% of the C57BL/6J mice that received injections of $10^{8.7}$ or more rickettsiae. Lesions were not observed in surviving A/J mice.

Mice of both strains that received at least one rickettsia developed significant antibody titers against phase I, phase II, and LPS antigens; titers increased with increasing rickettsial doses. At lower antigen doses, phase I and phase II antibody titers in the two mouse strains were not greatly different, but at doses above $10^{4.8}$ rickettsiae, C57BL/6J mice developed higher titers than A/J mice. Conversely, A/J mice developed higher LPS titers than C57BL/6J mice, at lower infective doses.

Thirty days after infection the WCI- and ConA-stimulated proliferative responses of spleen cells from each strain were compared for each level of infection. The responses of WCI-stimulated cells from C57/BL/6J mice were significantly (P <0.05) higher than those from A/J mice which did not survive higher doses. Stimulation indices for cells from C57BL/6J mice increased as the infecting dose increased, and peaked at a dose $10^{5.7}$ rickettsiae; responses were suppressed at higher doses. C57BL/6J mice injected with $10^{6.7}$ or more infectious *C. burnetii* exhibited stimulation indices no higher than those of cells from uninfected mice. Responses of WCI-stimulated cells from infected A/J mice peaked at a dose of $10^{2.7}$ rickettsiae. Higher infective doses significantly suppressed the proliferative responses of A/J cells to WCI stimulation. ConA-stimulated responses of cells from both strains were similar for infective doses below $10^{0.7}$, but, at higher doses, A/J cells were significantly suppressed compared to C57BL/6J mice. Compared with the response of cells from uninfected A/J mice, the mitogenic response of cells from infected A/J mice were suppressed at all infection levels. By contrast, the response of cells from infected C57BL/6J mice were significantly ($P < 0.05$) suppressed below control values only by infecting doses of $10^{6.7}$ or more rickettsiae; 7 logs more than the dose that suppressed the response in sensitive A/J mice.

Junin virus Junin virus, the causative agent of Argentine hemorrhagic fever, has been shown to be transmissible in small-particle aerosols, and exhibits a biological half-life approximating 28 min. A candidate vaccine strain (Candid #1) has been developed (Dr. J. Berrera-Oro, Virology Division) and is in the process of being evaluated for safety and efficacy. A study was designed, therefore, to evaluate the protective response induced by the Candid #1 strain versus airborne challenge.

The outbred guinea pig-Romero strain model was selected because of the data base that had been generated previously. Four groups of guinea pigs each were inoculated with decreasing concentrations of the Candid #1 strain. Each animal received a total volume of 0.5 ml of inoculum s.c. The airborne challenge was estimated to be an inhaled dose of 20,000 PFU or approximately $10^{3.8}$ LD_{50}. Control groups of guinea pigs were inoculated with diluent-virulent challenge, high dose Candid #1-sham challenge, diluent-sham challenge, or remained uninoculated. Rectal temperature and body weight evaluations were made 3 or 7 times per week throughout the study.

There were no deaths among those that received 30 or more PFU of the Candid #1 strain. Eleven of the guinea pigs in the lowest vaccine group (<1 PFU) survived. Each of the surviving guinea pigs exhibited a plaque reduction neutralization 80 titer of 8 or greater on day 60 after vaccination. None of the animals that died, low-dose vaccinees or controls, had detectable neutralizing humoral antibodies. Further, the mean time-to-death was essentially identical among the animals that died.

Surviving vaccinated guinea pigs appeared normal throughout the duration of the study. Rectal temperatures constantly remained within normal limits. Growth rates, as indicated by the body-weight determinations, were slowed slightly for 7 to 10 days after virulent challenge, but no dimunution in body weight was observed.

By contrast, unvaccinated control animals and vaccinees that died became febrile by day 7 to 9. Elevated temperatures, as high as 41.7°C, were maintained until just prior to death. Loss of body weight commenced with the onset of fever and continued until death. Average loss per guinea pig was about 15%.

Concentrations of virus in the tissues of animals at the time of death mimicked those observed at the terminal stages in previous pathogenesis studies. Virus was not detected in any tissue at any time point (15, 30, 45, 60 days) after challenge among surviving vaccinees. Further analysis will determine if the challenge virus can be detected in the hours immediately following challenge.

From these data, it readily can be concluded that the Candid #1 strain induced a protective response against a significant airborne challenge of virulent virus.

PUBLICATIONS:

1. **Berendt, R. F., and N. K. Jaax.** 1985. Effect of prior influenza virus infections on susceptibility of AKR/J mice to respiratory challenge with *Legionella pneumophila*. *J. Lab. Clin. Med.* 105:124-131.

2. **Williams, J. C., V. Sanchez, G. H. Scott, E. H. Stephenson, and P. H. Gibbs.** 1985. Variation in responsiveness of Balb/c sublines and congenic mice to phase I *Coxiella burnetii* infection and vaccination. *In* M. Potter (ed), Immunological response of the Balb/c mouse. Springer-Verlag, New York (In Press).

PRESENTATIONS:

1. **Berendt, R. F., J. Jemski, S. Leppla, A. Johnson-Winegar, and B. Ivins.** The use of toxin-components for the immunoprophylaxis of inhalation anthrax. Presented, 85th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, Mar 1985.

2. **Berendt, R. F.** Use of anthrax toxin components for immunization. Presented, USAMRIID Professional Staff Conference, Mar 85.

3. **Scott, G. H., and E. H. Stephenson.** A survey of inbred mice for sensitivity to Q-fever: Protective immune response in a Sensitivity Strain. Presented, Annual Meeting of the 84 American Society of Tropical Medicine and Hygiene, Baltimore, MD, Dec 84.

4. **Scott, G. H., and E. H. Stephenson.** Susceptibility of Inbred mice to *Coxiella burnetii* infection. Presented, American Society of Rickettsiology, Laguna Beach, CA, Feb 85.

5. **Scott, G. H., and E. H. Stephenson.** Susceptibility of inbred mice to *Coxiella burnetii* infection. Presented, 85th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, Mar 85.

6. **Wood, O. L.** Evaluation of various Rift Valley fever virus vaccines. Presented, USAMRIID Professional Staff Conference, Mar 85.

7. **Moeller, R. B.** Efficacy of the Austrian tickborne encephalitis virus vaccine. Presented, USAMRIID Professional Staff Conference, Mar 85.

8. **Stephenson, E. H.** Studies with Junin Virus Candid #1 strain. Presented, USAMRIID Professional Staff Conference, Mar 85.

LITERATURE CITED

1. **Jemski, J. V.** 1983. U.S. Army Medical REsearch Institute of Infectious Diseases Annual Progress Report, FY 83.

2. **Berendt, R. F.** 1984. U.S. Army Medical Research Institute of Infectious Diseases, Annual Progress Report, FY 84.

Table 1. Effect of time after vaccination on protection and serological titers of guinea pigs exposed to virulent *B. anthracis*.

Week	Treatment	Geom. mean titers (reciprocal)	% Survival
2	Saline	< 32	10
	Sterne	6,502	100
	MDPH	20,643	100
4	Saline	<32	0
	Sterne	1,780	100
	MDPH	14,596	70
8	Saline	<32	0
	Sterne	2,580	90
	MDPH	4,598	40
16	Saline	< 32	0
	Sterne	1,448	80
	MDPH	1,290	40
	Sterne Boost[a]	8,192	100
	MDPH[a]	14,263	90
27	Saline	< 32	0
	Sterne	1,448	50
	MDPH	1,290	30
	Sterne Boost[a]	3,251	100
	MDPH Boost[a]	3,251	40

[a]Boosted at 12 weeks.

Table 2. Effect of spore concentration (Sterne) on survival and serological titers.

Spores injected (CFU)	Survivors/ total	Percent survivors	Seroconversion[a] (%)	Serum antibody titer[b]
Control	0/23	0	0	< 32
2.5×10^2	1/24	4.2	4.2	32
2.5×10^3	2/21	9.5	16.1	45
2.5×10^4	2/23	8.7	25.0	181
2.5×10^5	7/22	31.8	58.3	558
2.5×10^6	22/24	91.7	100.0	24,549

[a]Antibody to PA.
[b]Reciprocal of geometric mean for animals that seroconverted.

Table 3. Immunizing efficacy of MDPH vaccine in A/J mice.

Treatment	Dead/total[a]	MDTD[b]
None (saline control)	10/10	2.2
MDPH:0.002 μg	10/10	2.6
MDPH:0.20 μg	10/10	2.2
MDPH:2.0 μg	8/10	3.2
MDPH:20 μg	5/10	2.3

[a]Challenge dose was 10 LD_{50} (aerosol) of the V-1-B strain.
[b]Geometric mean days to death.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 305649	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763A	3M263763D807	AJ	021
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Development of Detection and Treatment of Marine Toxin Poisoning

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	0.5	75
86	0.0	0*

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Medical Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Davio, S R
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7181
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Hewetson J F
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Parker, G W

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Saxitoxin; (U) BW Defense; (U) Military Medicine; (U) Lab Animals; (U) Mice; (U) Marine Toxins; (RAM I)

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop methods for field and laboratory detection of marine toxins in biological and environmental samples. To develop prophylactic and/or therapeutic treatments for toxin-exposed soldiers.

24. (U) Detection of marine toxins will be accomplished by immunoassays. Prophylactic/therapeutic agents will be based on polyclonal and monoclonal antibodies to these toxins. Stable toxin-protein conjugates will be developed and tested as possible vaccinating agents.

25. (U) 8410-8509-Enzyme-linked immunoassays have been developed for detection of STX with anti-STX rabbit and mouse sera. Assay sensitivities with these sera are 0.1 and 1.0 ng STX/ml, respectively. Rabbit polyclonal antiserum has been shown to dramatically reverse the effects of saxitoxin in the rat. Even rats with toxin-induced respiratory paralysis began breathing when given antiserum i.v. These results indicate a real potential for antiserum as a therapeutic agent. Rabbit polyclonal antiserum also protected against intratracheally injected STX in mice. Passive immunotherapy is probably viable approach for combating aerosolized STX.

*This research will be part of Work Unit 807AK for FY 86.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AJ-021: Exploratory Development of Detection and Treatment of Marine Toxin Poisoning

PRINCIPAL INVESTIGATOR: Stephen R. Davio, CPT, Ph.D.

ASSOCIATE INVESTIGATORS: John F. Hewetson, Ph.D.
Gerald W. Parker, CPT(P), DVM
Dale G. Martin, CPT(P), DVM, Ph.D.
Donald Creasia, Ph.D

Background:

Research in the past year has concentrated primarily on the marine neurotoxin - saxitoxin (STX). This toxin is produced by dinoflagellates of the genus *Gonyaulax* and by blue-green algae of the genus *Aphanizomenon*. STX is the most potent of a family of toxins (the so-called saxitoxins). The other toxins in this family are neosaxitoxin (an N-1 hydroxy derivative of STX) and gonyautoxins 1-4 (sulfated derivatives of STX and Neosaxitoxin).

STX has long been regarded a potential BW threat due to the following properties: (i) extreme toxicity (LD_{50} ~ 10μg/kg, mice, i.p.); (ii) speed of action (time-to-death < 10 min in mice injected i.p.); (iii) stability (toxin purified 20 years ago retains all of its toxicity); and (iv) potential for mass production by culturing the source organisms. For these reasons, STX has been the initial focus of our marine toxin studies. This annual report describes research aimed at the goals of detection and therapy for saxitoxin poisoning.

Summary:

Detection Enzyme-linked immunoassays have been developed for detection of STX. These assays use anti-STX rabbit serum or anti-STX mouse serum. The assay sensitivities with these sera are 0.1 ng STX/ml and 1.0 ng STX/ml, respectively.

Pathophysiology and Therapy Polyclonal rabbit antiserum was tested for its ability to reverse the pathophysiological effects of STX in the rat. The rabbit antiserum given i.v. was shown to dramatically reverse the severe hypotension associated with lethal doses of STX poisoning. Even more impressive was the finding that rats which had become apnic because of STX-induced respiratory paralysis began breathing spontaneously when given antiserum i.v.

In another study, mice pre-injected with antiserum were protected from a LD_{99} dose of STX injected intratracheally. These results may have significance for protection of troops from STX aerosol attack.

Progress:

Detection Immunoassays for STX have been developed under the direction of Dr. John F. Hewetson and Dr. Stephen R. Davio. These immunoassays are all based on the so-called "second antibody" format. In this type of assay, saxitoxin is detected by its ability to displace STX-specific antibodies from binding to STX-coated microtiter plates. The amount of STX-specific antibody bound to the plates is quantitated by adding a second antibody coupled to a peroxidase enzyme. The peroxidase substrate is added to induce color formation. Thus, the presence of saxitoxin in a sample will cause a decrease in color.

Our experiments have tested different ways of binding STX to plates. Plates were coated with STX coupled to keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), horse IgG, human IgG, and egg albumin with formaldehyde. These conjugates were prepared directly in the microtiter wells according to Davio et al. (1985). STX was also coupled to polylysine with fomaldehyde in a test tube, the sample was dialyzed, and then was loaded into microtiter wells according to the method of Chu et al. (1985). Several antisera were also tested, including two anti-STX rabbit sera and pools of anti-STX mouse sera. The best results were obtained with plates coated with STX-polylysine and with an anti-STX rabbit serum obtained from Dr. Patrick Guire's group at Bio-Metric Systems, Inc. (Carlson et al., 1984). The sensitivity of the ELISA with these components was ~ 10 to 100 pg STX/ml, making it the most sensitive assay yet for detection of saxitoxin. Good results have also been obtained with STX-polylysine reacted with anti-STX mouse antibodies. These components have resulted in an assay with a sensitivity of ~ 1.0 ng STX/ml. These assays are still in a preliminary stage. Future research will concentrate on finalizing assay conditions and testing them for detection of STX in biological fluids.

Pathophysiology and Therapy CPT Dale Martin and CPT Gerald Parker (both of Pathophysiology Division) recently completed studies in which they tested the ability of anti-STX rabbit serum to counteract STX in the rat. This study was a follow-up to that of Davio (1985) which showed that pre-injection with antiserum protected mice against lethal doses of STX. Martin and Parker's study was to address the question of whether antiserum could reverse the effects of STX.

Martin and Parker characterized a number of pathophysiological responses to STX poisoning in the rat. These included a dose-dependent bradycardia and hypotension, decreased minute volume, respiratory rate, and arterial oxygen tension. Eventually, high doses of STX (16μg/kg) led to apnea and death within 9 to 23 min. Rabbit antiserum administered i.v. after the onset of symptoms immediately reversed the effects of saxitoxin. Even in animals which had become apnic, i.v. administration of antiserum often led to spontaneous breathing without the assistance of artificial respiration.

Artificial respiration alone was also evaluated as a means of therapy for saxitoxin poisoning. Arterial blood pressure and heart rate remained severely depressed in all animals through the 2-h study period. Some animals were ventilated up to 5 h without any evidence of improvement in blood pressure. As a pilot study, one rat was ventilated 1 h after STX-induced apnea before treatment with antiserum. This regimen resulted in a more gradual return of cardiopulmonary values to normal values than when antiserum was given immediately after apnea.

In summary, STX antiserum reversed the cardiorespiratory depression observed after a lethal dose of i.p. administered saxitoxin. These results suggest that specific antisera can be developed as antidotes to reverse the pathophysiology induced by a sodium-channel blocking toxin.

Another study, conducted by Dr. Donald Creasia and CPT Davio, tested whether antiserum to STX could protect against STX injected intratracheally. This study was a first attempt to determine whether antiserum can protect against an aerosol exposure of STX. This study was conducted in two stages: (i) the lethality curve of STX injected intratracheally was determined, and (ii) mice were pre-injected with dilutions of antiserum i.p. and challenged 1 h later with an LD_{99} dose of intratracheal STX.

The LD_{50} for intratracheally injected STX was 8 to 16 μg/kg; 20 μgSTX/kg was an approximate LD_{99} dose. Mice pre-injected with 100 μl antiserum (1:16 dilution) did not survive an intratracheal challenge of 20 μg/kg 1 h later. However, mice pre-injected with lesser dilutions (i.e., higher concentrations) of antiserum showed an increasing tendency toward survival with increasingly more concentrated antiserum samples. This study supports the hypothesis that antiserum can protect against intratracheally injected saxitoxin and indicates that prophylactic use of antiserum may protect soldiers against an imminent saxitoxin aerosol attack.

PRESENTATIONS:

1. **Hewetson, J.F., S.R. Davio, and J.E. Beheler.** Progress toward development of monoclonal antibodies for identification and treatment of saxitoxin poisoning, 8th World Congress on Animal, Plant, and Microbial Toxins, Newcastle upon Tyne, England, 12-16 August 1985.

PUBLICATIONS:

1. **Davio, S. R.** 1985. Neutralization of saxitoxin by anti-saxitoxin rabbit serum. *Toxicon.* (In Press).

LITERATURE CITED

1. **Davio, S. R., J. F. Hewetson, and J. E. Beheler.** 1985. Progress toward development of monoclonal antibodies to saxitoxin: antigen preparation and antibody detection. Proceedings of the Third International Conference on Toxic Dinoflagellates. (In Press)

2. **Chu, F. S., and T. S. L. Fan.** 1985. Indirect enzyme-linked immunosorbent assay for saxitoxin in shellfish. *J. Assoc. Off. Anal. Chem.* 68:13-16

3. **Carlson, R. E., M. L. Lever, B. W. Lee, and P. E. Guire.** 1984. Development of immunoassays for paralytic shellfish poisoning, a radioimmunoassay for saxitoxin, pp. 181-192. *In* E. P. Ragelis (ed.), Seafood Toxins. ACS Symposium Series, No. 262.

4. **Davio, S. R.** 1985. Neutralization of saxitoxin by anti-saxitoxin rabbit serum. *Toxicon.* (In Press).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 305650	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 12 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63763D	3M263763D807	AK	022
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Development of Detection and Treatment of Mycotoxin Poisoning

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	2.0	269
86		

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathophysiology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Hewetson, J F
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7181
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (If available) Fricke, R F
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (If available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) BW Defense; (U) Military Medicine; (U) Mycotoxins; (U) Lab Animals; (U) Mice; (U) Diagnosis; (U) RAM I; (U) Monkeys

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop the ability to detect toxins in biologic fluids, to pursue studies on basic mechanism of action that directly relate to the development of potential therapy, and to develop prophylactic and/or post exposure therapy for soldiers exposed to toxins.

24. (U) Use immunoassays and develop alternate novel assays for detection of toxins and metabolites in biological specimens. Study basic toxicology and immunology including distribution, metabolism, and excretion in order to better develop therapeutic agents. Test a broad spectrum of agents both off the shelf and newly developed ones for prophylactic and/or post-exposure therapy, including drugs as well as vaccines and passive antibody transfer.

25. (U) The ability to detect T-2 toxin by an ELISA immunoassay has improved considerably. T-2 or its metabolite can be detected by immunoassay in organs of exposed animals. Saxitoxin can also be readily detected in immunoassay and monoclonals against saxitoxin have been developed. Studies on the response of animals that have been exposed to T-2 opportunistic organisms have been performed and are being further evaluated. Passive transfer of antibody can be used for post-exposure treatment of animals exposed to either saxitoxin or T-2 toxin. Several drugs have been shown to be effective in reducing the lethality of T-2 toxin. These include glutathione prodrugs, anti-inflammatory glucocorticoids, intestinal absorbing agents, anti-oxidants, and microsomal inducing agents. The time of delivery, before, during, or after, exposure to T-2 were critical for the optimal effect.

BODY OF REPORT

PROJECT NO. 3M263763D807: Exploratory Development of BW Vaccines/Drugs

WORK UNIT NO. 807-AK-022: Exploratory Development of Detection and Treatment of Mycotoxin Poisoning

PRINCIPAL INVESTIGATOR: John F. Hewetson, Ph.D.

ASSOCIATE INVESTIGATOR: Robert F. Fricke, CPT

Background:

T-2 mycotoxin is a potent non-protein toxin which can cause death or illness upon contact or ingestion. The need for rapid, reliable, and quantitative methods for detection of the low molecular weight toxin, and the development of antidotes for treatment of mycotoxin poisoning is well established. Methods available for detection include variations of the radio immune assay (RIA) and enzyme-linked immunoassay (ELISA). The ELISA technology is most appropriate for field use since it does not involve radioisotopes. Lethality of T-2 toxin exposure with the mouse animal model has been effectively reduced with glutathione prodrugs, anti-inflammatory glucocorticoids, intestinal adsorbing agents, anti-oxidants, and microsomal inducing agents. Adaptation of the ELISA technology for toxin detection and the defining of an effective therapy for the treatment of T-2 mycotoxin exposure are major goals of this work unit.

Summary:

Detection of T-2 mycotoxin by ELISA has been standardized by using three different sources of antisera; a mouse monoclonal, a rabbit polyclonal, and a goat polyclonal. All three work well but have different sensitivities. The monoclonal antisera can detect T-2 at 100 ng/ml, while the rabbit and goat sera have sensitivities ≥ 1 ng/ml. An ELISA for saxitoxin has also been standardized and can reliably detect saxitoxin at the level of 100 pg/ml. This assay is equal in sensitivity to the membrane binding assay and several orders of magnitude more sensitive than the mouse bioassay.

The detection of T-2 toxin by radioimmunology techniques (RIA) in organs of exposed animals has been studied and compared to detection using radiolabeled tracers. The levels detected by RIA compare favorably with those found with tracer methodology.

The RIA for T-2 in blood and urine has been used to review the statistical basis for determining the false negative rates. This technique allows us to determine the exact dose that can be detected, with a precise determination of the probability a positive sample will be positive, or the probability that a positive sample would appear negative at any dose level. This has wide application in the analysis of potentially contaminated material, where it must be known exactly the dose that can be detected and the probability of calling a sample positive that is truly negative.

The affinity purification of goat anti-T-2 antisera has proceeded to a point where we are ready to do comparative tests with the available monoclone for

suitability in the T-2 RIA and ELISA. This affinity-purified antisera will also be used for treatment protocols and compared with a T-2 monoclonal antibody for possible therapeutic use.

We are using a mouse model with *Candida albicans* to study the possible synergistic effect of T-2 toxin and opprotunistic infections in experimental animals. We have found no difference in lethality due to *C. albicans* between mice exposed to a sublethal oral dose of toxin and unexposed control mice. However, with multiple exposure to a sublethal toxin dose, there is increased lethality in mice, presumably due to infection by *C. albicans*. Furthermore, isolation of viable organisms from organs of exposed animals is higher in the toxin-exposed group than in controls.

With the mouse animal model, several drugs have been shown to be effective in reducing the lethality of T-2 toxin. Significant protection was achieved with glutathione prodrugs, anti-inflammatory glucocorticoids, intestinal adsorbing agents, antioxidants, and microsomal inducing agents. Glutathione prodrugs were effective only when given prior to toxin. Both anti-inflammatory glucocorticoids and activated charcoal were effective in reducing lethality when administered both before and after toxin exposure. Some antioxidants, vitamin E and BHT, were found to be effective only when given prior to toxin exposure; while others - vitamin C, n-propyl gallate, ethoxyquin, and ascorbyl palmitate - were effective when given at the same time as toxin exposure. Microsomal inducing agents were effective only with daily pretreatment for three days.

Progress:

T-2 Toxin Detection - ELISA Considerable effort this year has been allocated to standardization of an ELISA for T-2 toxins. Previous efforts, reports in the literature, and contractor progress reports have all reported variable results and reproducibility problems with this assay. Factors that have been identified as critical include:

1. Proper concentration of antigen coated on the solid phase.
2. Proper carrier for hapten antigen.
3. Proper solid phase.
4. Critical concentration of first and second antibody.
5. Source (manufacturer) of the second antibody.
6. Proper blocking agent after antigen coating in solid phase.
7. Source of first antibody.

The proper combination of the above has resulted in an assay that is reliable and sensitive. We are able to detect T-2 using the 15H6 monoclonal antibody at 1 ng/ml. This is a thousand-fold more sensitive than previous reports with this antibody. The significant difference appears to be the use of T-2 conjugated to polylysine as the solid phase rather than BSA, as had been used previously. This sensitivity is the same as that obtained with rabbit and goat polyclonal sera against T-2 when assayed on T-2-polylysine.

Saxitoxin Detection - ELISA The detection of saxitoxin by competition-ELISA techniques has been standardized in a manner similar to that taken with T-2. The same critical factors apply. We have found that saxitoxin conjugated to polylysine and coated to a polyvinyl chloride solid phase is an excellent antigen for anti-saxitoxin binding and saxitoxin detection. We have used three sources of anti-saxitoxin serum: (a) rabbit anti-saxitoxin sera prepared against STX-BSA by Dr. Chu; (b) rabbit anti-saxitoxin sera prepared by Dr. Guire against STX-BSA; and (c) a pooled mouse immune sera prepared against STX-KLH by Dr. Davio. Sera (b) and (c) give reproducible assays, with sensitivities between 100 pg and 1 ng/ml. There are still technical problems with antisera (a).

T-2 Detection in Organs The distribution of T-2 and its metabolites in organs of experimental animals has been studied previously by using radiolabeled T-2. This technique is unworkable with specimens obtained in a "natural" exposure. Therefore, we initiated a study to determine if T-2 can be detected in organs by an immunoassay. Since the only antisera that are currently available are against T-2, we were limited to detection of this primary toxin and HT-2 (which gives a 10% cross reactivity). T-2 is metabolized quickly, especially in the liver, so that only a small fraction of the original toxin would be expected to be present.

Rats were inoculated s.c. with 3 mg/kg of T-2 toxin. One hour later they were killed, and the liver, heart, spleen, and kidney were removed and quick-frozen in liquid nitrogen. Aliquots from each tissue were macerated in liquid nitrogen with a mortar and pestle, and homogenized and extracted three times in 100% methanol. The samples were evaporated to dryness and resuspended in 10% methanol - phosphate-buffered saline. The extracts were then assayed for T-2 toxin with a standard radioimmunoassay. For comparison purposes, a second group of rats was inoculated with ^{3}H-labeled T-2 and the organs harvested and extracted as described above. These tissues were assayed for T-2 and its metabolites by scanning for radioactivity with a Bioscan BID 100 radioisotope scanner.

The results of these studies indicated that T-2 and T-2 cross-reacting metabolites (HT-2) could be detected and quantitated with the competitive RIA in all organ extracts. There was fairly good agreement between recovery measured by the RIA and that measured by the radiochromatography method. Other metabolites that can be measured and quantitated by the Bioscan cannot be detected with the antibody method. Thus, the RIA assay will be appropriate for determining an acute exposure to T-2 toxin and a semi quantitation of the amount of exposure. RIA of metabolites after a long-past exposure is not yet possible.

T-2 Detection - RIA Standardization In order to set standards for false negative rates and false positive rates in the immunoassays, the RIA for T-2 toxin was selected as a prototype, since this assay works well in our hands and yields reproducible results. The data were analyzed by an inverse prediction method developed by P. Feder under contract to the Army. The models and tests of significance were obtained by SAS GLM. These techniques enabled us to (1) estimate, with 95% confidence intervals, the concentration in a given sample; (2) estimate the precision of an assay; (3) estimate the false negative rate; and (4) estimate the detection limit based on the false negative rate. Standard curves for T-2 in blood and urine were constructed and analyzed by the above technique. We showed that T-2 can be reliably detected in blood and urine with detection limits of 3.5 ng/ml and 10.3 ng/ml, respectively, with a false negative rate of 10%. This means, for example, that the probability of calling a blood sample that actually contains 3.5 ng of T-2 toxin negative is 10%.

<u>Treatment of T-2 Toxin Poisoning</u> Although there are no known specific antidotes for treatment of T-2 mycotoxin poisoning, recent findings indicate that several different classes of drugs are effective in reducing the lethality of this toxin. The drugs that have shown positive therapeutic effects are anti-inflammatory steroids, glutathione prodrugs, intestinal adsorbing agents, microsomal inducing agents, and antioxidants/free radical scavengers.

The anti-inflammatory glucocorticoids: dexamethasone, prednisolone, methyl prednisolone, and hydrocortisone, are highly effective in reducing the lethality of T-2 toxin. Of the steroids tested (at equally effective doses), dexamethasone showed the highest effectiveness, followed by prednisolone > methylprednisone > hydrocortisone. Dexamethasone in combination with either prednisolone or methylprednisolone did not increase the survival rate. Dexamethasone was highly effective when given either before, during, or after exposure to T-2 toxin. Treatment 60 min prior to toxin exposure resulted in the greatest survival rate, with decreasing effectiveness at 60, 120, and 180 min post-exposure.

Antioxidants, vitamin E and butylated hydroxytoluene (BHT), were effective only with daily pretreatment for three days prior to toxin exposure. Other antioxidants: vitamin C, ascorbyl palmitate, ethoxyquin, and n-propyl gallate, were all effective when administered at the same time as toxin exposure. Of these antioxidants, vitamin C showed the highest efficacy in terms of decreased lethality and relative risk; and increased mean survival times, mean time-to-death, and relative potency, as summarized in Table 1.

<u>Current Projects</u> Two ongoing projects are the affinity purification of goat anti T-2 and the effect of T-2 on opportunistic infections. Preliminary results indicate that we have affinity-purified the antibody. ELISA results have shown a positive binding to antigen and this binding could be inhibited by free T-2. Further work is needed to characterize this purified antibody.

We have used *Candida albicans* infection on mice as one model system to study the synergistic effect of opportunistic infection and T-2 toxin. Mice were exposed to toxin in propyl glycol by an oral route.

Candida was injected i.v. or i.p. at graded concentrations. In selected experiments, the organs from animals exposed to *Candida* or *Candida* plus toxin were removed, macerated, and plated to determine the concentration of infectious organisms.

When mice were dosed with a sublethal acute dose of T-2 followed by *Candida albicans*, there was no apparent difference between the deaths in the experimental group and control (*Candida* alone).

PRESENTATIONS:

1. **Hewetson, J. F.** USAMRIID Staff Conference, Fort Detrick, Frederick, MD, November 1984.

PUBLICATIONS:

1. **Fricke, R. F.** 1985. Effect of glucocorticoid treatment on lethality of T-2 mycotoxin in mice. *The Toxicologist* 5:205.

2. **Hewetson, J. F., J. E. Beheler, and J. G. Pace.** 1985. Detection of mycotoxin in organs of exposed animals by immunological techniques. *Fed. Proc.*

3. **Hewetson, J. F., S. R. Davio, and J. E. Beheler.** 1985. Progress toward development of monoclonal antibodies for identification and treatment of saxitoxin poisoning. Proceedings, 8th World Congress on Animal, Plant, and Microbial Toxins.

4. **Fricke, R. F.** 1985. Protective effects of anti-inflammatory agents against T-2 mycotoxin poisoning. Proceedings, 8th World Congress on Animal, Plant, and Microbial Toxins.

Table 1 Effectiveness of Vitamin C in Treatment of T-2 Mycotoxin-exposed Mice

	Vitamin C (mg/kg)			
	0	400	800	1200
Survival Time (h)	34	115	133	124
Time to Death (h)	21	36	51	59
% Lethality	100%	40%	30%	40%
Relative Risk	1.00	0.183	0.118	0.160
Relative Potency	1.00	1.30	1.39	1.23

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 303917	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750D	3M463750D809	AC	001
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code) Vaccine, Advanced Development

12. SUBJECT AREAS 0613 Microbiology; 1503 Defense; 0601 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 04	89 01	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	2.0	621
86	2.0	131

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Airborne Diseases Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Stephenson, E H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Williams, J C
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Q Fever (U) Coxiella burnetii; (U) Vaccines; (U) Medical Defense; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) *Coxiella burnetii*, the causative agent of Q fever, is considered by most experts to have those properties which make it a potential candidate for BW. The existing vaccine to protect US troops against this threat is reasonably effective, but causes sterile abscesses in previously sensitized individuals. This institute has a broad program to improve the current vaccine.

24. (U) This work unit is dedicated to the transfer of research-level vaccine production technology to conditions for pilot-scale processing of the Q fever vaccine. Definition of the pilot-scale conditions will permit the orderly production of large volume lots of vaccine.

25. (U) 8410-8509 - Mass-scale purification and chemical extraction procedures for the commercial production of Q fever vaccine were improved to meet the unique conditions of large volume operations. Through collaborative efforts, personnel at the plant of the contractor responsible for pilot-scale production were trained in the specific and unusual aspects of procedures using *Coxiella burnetii*. The pilot lot of Q fever vaccine now is being produced.

BODY OF REPORT

PROJECT NO. 3M463750D809: Drug and Vaccine Development for Medical Defense Against BW

WORK UNIT NO. 809-AC-001: Vaccine, Advanced Development

PRINCIPAL INVESTIGATOR: E. H. Stephenson, D.V.M., Ph.D., COL, VC

ASSOCIATE INVESTIGATOR: J. C. Williams, Ph.D., CDR, USPHS

Background:

Coxiella burnetii, the causative agent of Q fever, is considered by most experts to have those properties which make it a potential candidate for BW. The existing vaccine to protect US troops against this threat is reasonably effective, but causes sterile abscesses in previously sensitized individuals. This institute has a broad program to improve the current vaccine.

This work unit is dedicated to the transfer of research-level vaccine production technology to conditions for pilot-scale processing of the Q fever vaccine. Definition of the pilot-scale conditions will permit the orderly production of large volume lots of vaccine.

Summary:

Mass-scale purification and chemical extraction procedures for the commercial production of Q fever vaccine were improved to meet the unique conditions of large volume operations. Through collaborative efforts, personnel at the plant of the contractor responsible for pilot-scale production were trained in the specific and unusual aspects of procedures using *Coxiella burnetii*. The pilot lot of Q fever vaccine now is being produced.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 303505	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY U	6. WORK SECURITY U
7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT	

10. NO./CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750D	3M463750D809	EA	001
b. CONTRIBUTING				
c. CONTRIBUTING	"NONE"	01		

11. TITLE (Precede with Security Classification Code) Drug and Vaccine Development/Rapid Identification and Diagnosis

12. SUBJECT AREAS 0613 Microbiology; 1503 Defense; 0601 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 03	88 01	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE | EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE | d. AMOUNT
e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	0.2	236
86	0.2	50

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Disease Assessment Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR LeDuc, J W
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7244
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Meegan, J
MILITARY/CIVILIAN APPLICATION H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Nuzum, E

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Vaccines; (U) Medical Defense; (U) Viral Diseases; (U) Immunological Reagents; (U) Antigens; (U) Rapid Diagnosis; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To conduct field tests of developed, rapid diagnosis assays. To assess availability and suitability of commercial reagents for use in rapid detection assays for common respiratory viruses affecting US military personnel. To support extramural contracts in rapid diagnosis.

24. (U) Develop immunoassays for respiratory viruses and potential BW agents. Evaluate selected assays in field laboratories. Supply contractors with standardized reagents, safety-tested and efficacy-tested, for use in development of rapid diagnosis systems.

25. (U) 8410-8509-Evaluation of commercially available reagents for rapid diagnosis of common respiratory pathogens was completed. Assays for influenza A and B, parainfluenza, adeno, and respiratory syncytial viruses were shown to accurately react with cell culture-propagated virus, but not with unamplified samples. A diagnostic kit has been developed that contains the best reagents from commercial sources and that uses an improved protocol. This technology has been transferred to Brooks Air Force Base, where a global respiratory virus surveillance program is ongoing. There, the kit will be further evaluated by using fresh clinical specimens. Throughout the year reagents were supplied to extramural contractors developing rapid diagnostic assays for military use for delivery of protective assays for 6 different pathogens. Prototype assays from 9 contracts were evaluated; the most promising were selected for further development. A rapid assay for sandfly fever was developed and evaluated in conjunction with a human-use, antiviral drug protocol. Viral antigen and viral-specific IgM was detected in all sera from infected patients and were absent in control sera. This demonstrated the ability of the test to rapidly diagnose a militarily important febrile disease.

BODY OF REPORT

PROJECT NO. 3M463750D809: Drug and Vaccine Development for Medical Defense Against BW

WORK UNIT NO. 809-EA-001: Drug and Vaccine Development/Rapid Identification and Diagnosis

PRINCIPAL INVESTIGATOR: James W. LeDuc, LTC, Ph.D.

ASSOCIATE INVESTIGATOR: James M. Meegan, CDR, USN, Ph.D.

Background:

The objectives of this study are to conduct field tests of rapid diagnostic assays developed at USAMRIID for agents of biological warfare potential or agents of geographic importance, to determine if commercially available reagents can be used for rapid diagnosis of common respiratory viruses; and to provide technical base support and specialized reagents for extramural contracts for development of rapid diagnostic assays against agents of biological warfare potential. The main technology utilized is the enzyme immunoassay, including several modifications of the fundamental test. Field tests were conducted in areas of the world where the viruses in question naturally occur and where select populations are known to be at risk of disease. The overall purpose of this work unit is to transfer assays developed in the laboratory or commercial sector to field use and evaluate their utility under realistic conditions.

In previous years, successful evaluation of DoD assays was conducted in Southwest and Southeast Asia by using epidemiologically relevant samples. Assays to detect rapidly virus-specific immunoglobulin M (IgM) were successful in detecting Rift Valley fever, Crimean-Congo hemorrhagic fever, and Chikungunya virus infections. In regard to respiratory viruses, most, but not all, commercial reagents functioned well in laboratory detection of respiratory viruses, including influenza, parainfluenza, adeno, and respiratory syncytial viruses. This facilitated preparation of an assay for use with our collaborators at the Brooks Air Force Base in their Project Gargle, a global surveillance program for respiratory diseases among Air Force personnel.

Previous year's support of extramural rapid diagnosis contracts included providing specific antibodies and inactivated antigens for use in development of new technology for rapid diagnosis.

Summary:

Evaluation of commercially available reagents for rapid diagnosis of common respiratory pathogens was completed. Assays for influenza A and B, parainfluenza, adeno, and respiratory syncytial viruses were shown to accurately react with cell culture-propagated virus, but not with unamplified clinical samples from acutely ill patients. A diagnostic kit has been developed that contains the best reagents from commercial sources and an improved protocol. This technology has been transferred to Brooks AFB, where a global respiratory-virus surveillance program is ongoing. There, the kit will be evaluated further by using fresh clinical specimens. Throughout the year, reagents were supplied to extramural contractors developing

rapid diagnostic assays for military use for delivery of protective assays for six different pathogens. Prototype assays from nine contracts were evaluated extensively; the most promising were selected for further development. A rapid assay for sandfly fever was developed and evaluated in conjunction with a human-use, antiviral drug protocol. Viral antigen and virus-specific IgM were detected in all sera from infected patients and were absent in control sera. This demonstrated the ability of the test to rapidly diagnose a militarily important febrile disease.

Progress:

This work unit encompasses three areas of study: (1) the field testing of DoD-developed, rapid diagnostic assay systems; (2) the evaluation of commercially available reagents for use in rapid diagnostic assays (especially for respiratory agents); and (3) the establishment of a technical base to support extramural contracts developing novel rapid diagnostic systems.

Whenever there is an opportunity, assays developed and optimized under Work Unit No. 807-AH-017 are tested (with funds from the work unit) under 'real life' conditions. In previous years this has included transport of the assay kits to OCONUS field sites in lesser developed countries for evaluation with human samples obtained from patients experiencing local endemic or epidemic diseases. In general, the tests have proved to be very durable, fast, reproducible, and simple. However, sensitivity of antigen-detection assays must still be improved. All rapid diagnostic systems tested were enzyme-linked immunosorbent assays (ELISA), which afford the advantages of simplicity, speed, reliability, and sensitivity. Our main goal is to detect antigen in clinical or environmental samples, and these assays can accomplish this in 2 to 3 h. However, we recognize that the first indication that a disease is present might be an infected serviceman. Consequently, for each agent, an attempt was made to include a rapid antibody assay which would establish a diagnosis, even if the patient was sampled after the viremic (antigenemic) phase, during the early convalescent stages when development of virus-specific IgM antibody is in progress.

We have also completed studies showing that these assays can successfully detect antigen in environmental samples, such as impinger air samples collected from battlefields during military exercises, and infected arthropod samples collected from the environment.

A unique opportunity to obtain and test antigen containing human sera was presented in a human-use antiviral drug trial. Volunteers infected with sandfly fever virus, Sicilian strain, were studied during their induced illness at USAMRIID. Daily sera were analyzed in an antigen-detection ELISA system which yielded results three h after the sera were drawn. Four patients became clinically ill, and in each case, the ELISA successfully detected viremia in sera drawn during the febrile period. Preliminary results were extended to include samples drawn from patients for up to one year post-illness. In addition to successfully detecting antigen in clinically ill soldiers, the antibody-detection ELISA successfully detected the early appearance of IgM antibody (just after the antigenemia stage of illness), and the somewhat later appearance of IgG antibody. Consequently, the disease can be rapidly diagnosed at any time during a 6- to 7-day period, either during the viremic clinical illness (2 to 3 days) or in the 3 to 4 days just post-illness when the patient displays high IgM but no IgG antibody.

As outlined last year, our investigations evaluating commercially available reagents for use in antigen-detection assays of respiratory virus have been less promising than the above reported results. We have tested a variety of reagents for use in ELISA systems for the detection of the respiratory viruses influenza A and B, parainfluenza 1 and 3, adenovirus, and respiratory syncytial virus. There has been considerable variation between manufacturers, and in some cases, between reagent lots. The best reagents from the most reliable sources have been selected and employed to develop air-improved, antigen-detection ELISA systems. Numerous investigators have reported studies attempting to detect respiratory viral antigens in clinical samples from patients ill with these viruses. In general, the systems have been universally insensitive, and the usefulness of the rapid assays was questionable. Our systems readily detect virus in prepared virus stocks, and in air-impinger samples to which stock virus has been added. However, our results with clinical samples have paralleled reports in the literature and reveal that these assays are insensitive and affected by fluctuating, nonspecific, background reactions. Additionally, it appears that antigen circulating in sera is not high-titered, or indeed common, in most of these respiratory diseases. The best source of antigen is nasopharyngeal samples.

The assays we developed and optimized for respiratory agents were tested in parallel with tests developed by investigators in Europe. In all cases, our assays were more sensitive than those utilized commonly in Europe. Our technology was then transferred to Brooks AFB for testing in Project Gargle, an ongoing DoD survey of respiratory diseases in Air Force personnel. The test successfully detected virus in media after virus in the clinical sample had been allowed to grow or amplify in cell culture. However, direct detection of viral antigen in clinical samples obtained without amplification in cell culture from acutely ill patients was not successful. These results are in agreement with the majority of similar studies attempting to rapidly diagnose respiratory viral infections. During the upcoming respiratory disease season, our collaborators will evaluate this assay on freshly collected samples. Unless unforeseen breakthroughs occur in private sector studies, these assays will be stored in their current format for potential future use.

During previous years our emphasis was on developing a strong technical base to help support DoD extramural contracts in rapid diagnosis. Support was provided in a number of areas. Most notable has been supplying quality reagents for contractors. Our program routinely develops, purifies, and labels with enzymes or biotin: (1) immunoglobulins of polyclonal and monoclonal origin, (2) viral antigens from a variety of sources, and (3) cloned DNA probes. Standardization studies were performed on each reagent to determine specificity, sensitivity, and suitability for diagnostic systems. Additionally, methods were established for inactivating infectious agents in such a way that safety is assured and the reactivity of the reagent is optimized. Whenever possible, suitable reagents were sought from collaborators; however, many reagents were prepared in-house. Reagents were collected, expanded, purified if needed, tested for reactivity, and shipped to appropriate contractors. Stocks of reagents have been inventoried, distributed into useful aliquots, and stored under optimal temperature conditions.

During this last year, a major effort was focused on testing the prototype kits developed by nine extramural contractors. Eight contractors supplied diagnostic kits to detect Rift Valley fever and Venezuelan encephalomyelitis viruses. Three contractors furnished *Coccidoides immitis* detection kits. Each kit was evaluated by four to eight individuals, all of whom were experienced in performing rapid diagnostic assays. During initial testing, all individuals rated such test performance characteristics as clarity of directions, ease of assay setup, and ease of interpreting results. All kits were first tested for their ability to detect undiluted antigens. Later tests included evaluations of the kit's sensitivity and specificity.

Many of the details of each contractor's tests are proprietary and cannot be reviewed herein. Briefly, the results of our evaluations indicated that about two-thirds of the kits could detect homologous antigen. However, only a few were as sensitive as a standard ELISA, and about one-third of the kits showed substantial nonspecific reactions. A detailed report was generated and provided to the Source Selection Board for 'Biological Agent Identification and Diagnostic System, Rapid, Field.' These data helped that group decide on contractors for continuation into phase II of development.

Finally, members of our program have served, almost daily, as technical consultants to the DoD personnel administering extramural contracts. They have participated in Source Selection Boards, reviewed contracts and quarterly reports, and helped formulate strategy dealing with which infectious agents should receive priority for study. Each of these efforts will continue during the expanded extramural rapid diagnosis contract program envisioned for the next two years.

PRESENTATIONS:

1. **Meegan, J. M., and J. W. LeDuc.** Successful use of rapid viral diagnostic assays in lesser developed countries. Presented, National Academy of Sciences Institute of Medicine joint meeting with the Board on Science and Technology for International Development of the National Research Council. May, 1985.

2. **MacDonald, C., K. McKee, J. Huggins, J. Morrill, J. Meegan, C. J. Peters, and P. Canonico.** Ribavirin prophylaxis of sandfly fever - Sicilian infection in human volunteers. Presented, Interscience Conference on Antimicrobial Agents and Chemotherapy. September, 1985.

3. **Tammariello, R., M. Faran, J. Meegan, and C. Bailey.** Application of ELISA for detection of Rift Valley fever virus in mosquitoes. Presented, Annual Meeting of the American Society of Tropical Medicine and Hygiene. December, 1984.

4. **Nuzum, E.** Development of a rapid viral antigen detection ELISA for respiratory viruses. Presented, Staff Conference, Brooks AFB. August, 1985.

5. **Meegan, J., L. Laughlin, and N. Birgis.** Enzyme immunoassay detection of IgM and IgG antibodies to Rift Valley fever in cerebrospinal fluid. Presented, 6th International Congress of Virology. September, 1984.

6. **Meegan, J. M.** Evaluation of diagnostic kits produced by phase I contracts under identification and diagnostic system biological agent, rapid, field (IDSBARF) program. Presented, IDSBARF Source Selection Board. July, 1985.

PUBLICATIONS: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 305651	2. DATE OF SUMMARY: 85 10 01	REPORT CONTROL SYMBOL: DD-DR&E(AR) 636				
3. DATE PREV SUM'RY: 84 10 01	4. KIND OF SUMMARY: D. CHANGE	5. SUMMARY SCTY: U	6. WORK SECURITY: U	7. REGRADING	8. DISB'N INSTR'N: CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D809	AN	002
b. CONTRIBUTING				
c. CONTRIBUTING	Cards			

11. TITLE (Precede with Security Classification Code) (U) Vaccine, Advanced Development

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	1.0	337
86	1.0	299

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Medical Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Cosgriff, T M
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2997
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Meadors, G F g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Vaccines; (U) Volunteers; (U) Phase I and II Clinical Trials; RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Conduct Phase I (safety and tolerance) and Phase II (efficacy) testing of experimental vaccines for prophylaxis of diseases of unique military importance, particularly those with potential as biological warfare threats.

24. (U) Experimental vaccines which have undergone immunogenicity and safety testing in preclinical studies will be evaluated in rigorous clinical trials after extensive scientific and ethical review.

25. (U) Studies of the Western equine encephalitis vaccine have been completed. No further testing of this vaccine will be undertaken because of the low probability for use of WEE virus in biological warfare. The vaccine will continue to be made available to persons working with the virus. Studies of the current Q-fever vaccine have been limited by the high rate of skin test positivity encountered to date at USAMRIID. Analysis of the correlation between skin test reactions, serology, and lymphocyte transformation are being evaluated in study volunteers. Studies on the Rift Valley fever vaccine are currently in progress. All of the above vaccines appear to be safe and immunogenic, with the exception of RVF vaccine lo 7, which produced local reactions.

BODY OF REPORT

PROJECT NO. 3M463750D809: Drug and Vaccine Development for Medical Defense Against BW

WORK UNIT NO. 809-AN-002: Vaccine, Advanced Development

PRINCIPAL INVESTIGATOR: Thomas M. Cosgriff, M.D., LTC, MC

ASSOCIATE INVESTIGATOR: Gilcin F. Meadors, M.D., MAJ, MC

Background:

As part of the task of developing vaccines to meet the biowarfare threat, Phase I and Phase II clinical trials of candidate vaccines are undertaken by the Medical Division. If these trials demonstrate that a vaccine is safe and immunogenic, Phase III studies are conducted in larger numbers of volunteers, and when possible, in endemic areas.

Summary:

Candidate vaccines currently undergoing clinical trials include vaccines against Rift Valley fever, Western equine encephalitis, and Q-fever. Other vaccines which are nearing clinical testing include vaccines against tularemia, Venezuelan equine encephalitis, Chikungunya, Argentine hemorrhagic fever, and a newer, improved vaccine against Q-fever.

During the current year, 41 volunteers completed a Phase I and II study of lots 10-18 of the Rift Valley fever vaccine, 25 volunteers began a year-long Phase I and II study of the Western equine encephalitis vaccine, and 25 volunteers completed a Phase I and II study of the current Q-fever vaccine.

All vaccines so far have been found to be safe and immunogenic, with the exception of lot 17 of the Rift Valley fever vaccine which produced large local reactions in 3 of 4 recipients. Response to the Western equine encephalitis vaccinewas similar, whether subjects received 2 or 3 immunizations. Only 3 of the subjects in the Q-fever vaccine trial were skin test negative and were therefore immunized. Analysis of the correlation among skin test reaction, serology, and lymphocyte transformation is ongoing.

Progress:

As the above summary indicates, considerable progress has been made in the clinical testing of several candidate vaccines. The Western equine encephalitis vaccine will not undergo further testing because the virus has little potential for use in biological warfare. Clinical studies of the Rift Valley fever vaccine will continue for the next several years. This vaccine appears not only to be immunogenic, but also to be protective, based on experience in laboratory workers.

PUBLICATIONS:

A manuscript on the initial clinical trials of the Rift Valley fever vaccine is in preparation.

PRESENTATIONS: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 305993	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 12 26	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D809	BA	004
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS			

11. TITLE (Precede with Security Classification Code) (U) Ribavirin

12. SUBJECT AREAS
0605 Clinical Medicine; 1503 Defense; 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE | EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE | d. AMOUNT
e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	0.2	371
86	0.2	78

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Canonico, P G
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2290
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Huggins, J W
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Linden, C D

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Ribavirin; (U) Antiviral Drug; (U) Sandfly Fever; (U) Virus; (U) Prophylaxis; (U) Treatment; (U) RAM I; (U) Volunteers

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop the drug ribavirin as an antiviral for treatment of viral diseases of military importance.

24. (U) Perform clinical trials to evaluate efficacy and toxicity in accordance with regulatory requirements.

25. (U) 8410-8509-Ribavirin has been evaluated in volunteers in a prospective prophylactic study against sandfly fever virus. An oral dose of 400 mg (t.i.d.), given for 6 days beginning 1 day before exposure, was effective in preventing illness. Statistically significant changes were limited to the placebo group and reflected typical responses to a viral infection including fever and leukopenia. There were no clinically significant differences between the placebo and ribavirin-treated subjects with regard to red cell parameters, clinical chemistries, and urinalysis. Although drug-treated subjects remained asymptomatic, four of six seroconverted by day 28. Ribavirin, therefore, prevented sequelae but did not prevent an immunological response to sandfly fever virus.

BODY OF REPORT

PROJECT NO: 3M463750D809: Drug and Vaccine Development for Medical Defense Against BW

WORK UNIT NO: 809-BA-004: Ribavirin

PRINCIPAL INVESTIGATOR: Peter G. Canonico, Ph.D.

ASSOCIATE INVESTIGATORS: John W. Huggins, Ph.D.
Carol D. Linden, PH.D.
C. J. Peters, MD, COL, MC
James Meegan, Ph.D., CDR, USN
Carolyn MacDonald, MD. CPT. MC
Kelly T. McKee, Jr., MD, MAJ, MC
Lauren Reed, MAJ, MSC
Paul Gibbs
Dwayne D. Oland

Background:

Although most viral diseases are not associated with high mortality and are self-limiting, certain disease outbreaks, such as the influenza pandemic of 1918-19, can be associated with great loss of life. We know very little about exotic viral diseases with which we are presently unable to cope (1). Many viruses with the potential for inducing illness of high morbidity and mortality remain endemic in certain areas of the world; notable examples of these viral diseases are Rift Valley fever, Lassa fever and Ebola in Africa, Argentinian and Bolivian hemorrhagic fevers in South America, and Korean hemorrhagic fever in Asia. Numerous other examples can be cited easily. Broad-spectrum antiviral agents would be welcome insurance against the threat of virus outbreaks during military operations.

Isatin-β-thiosemicarbazone, amantadine, adenine arabinoside, and, recently, acyclovir, have been approved by the Food and Drug Administration for the prevention or treatment of virus-induced disease (2). Isatin-β-thiosemicarbazone and amantadine are approved principally for prophylactic use against smallpox and A-2 influenza, respectively. Adenine arabinoside has been approved for the treatment of primary genital herpes infections. These compounds have extremely narrow activity spectra. In contrast, the new antiviral drug ribavirin appears to provide broad-spectrum activity (3). It was the purpose of this protocol to evaluate in humans the potential of ribavirin to prevent sandfly fever.

Summary:

Ribavirin has been evaluated in volunteers in a prospective prophylactic study against sandfly fever virus. An oral dose of 400 mg (total infectious dose - t.i.d.), given for 6 days beginning 1 day before exposure, was effective in preventing illness. Statistically significant changes were limited to the placebo group and reflected typical responses to a viral infection, including fever and leukopenia. There were no clinically significant differences between placebo- and ribavirin-treated subjects with regard to red cell parameters, clinical chemistries, and urinalyses. Although drug-treated subjects remained asymptomatic, four of six seroconverted by day 28. Ribavirin, therefore, prevented sequelae but did not prevent an immunological response to sandfly fever virus.

Progress:

Subjects were selected from Medical Research Volunteer Subject (MRVS) personnel. Individuals were interviewed personally, given an opportunity to ask questions, and to express their desires regarding participation in the study. Three female and thirteen male subjects, 20- to 35-years-old, were selected from among those who volunteered to participate in the study.

On admission to the study, subjects were given a physical examination, and clinical laboratory tests were performed. All subjects were found to be in good health.

Ten of the 16 volunteers for the study had been immunized previously with Rift Valley fever (RVF) vaccine. Since it was not known if prior immunization with a related virus would alter susceptibility of subjects to sandfly fever virus infection, subjects were randomized to the virus challenge groups based on known prior vaccination to RVF. Four of the ten RVF-vaccinated subjects were selected to participate in group III, the pharmacokinetic phase of the study. The remaining six RVF-vaccinated subjects were assigned to the placebo (group I) and drug-treated (group II) by a computerized randomized process. The six non-RVF immune subjects were assigned to groups I and II by a similar process. The composition of the three experimental groups were as follows:

	ID#	Sex	RVF Vaccination
Group I	459-37-7786	M	No
Placebo	301-76-9295	M	No
+ SFF	519-90-0553	F	No
	233-72-6929	M	Yes
	393-70-1867	M	Yes
	325-58-3805	M	Yes
Group II	092-56-8905	M	No
Ribavirin	254-37-2962	M	No
+ SFF	161-48-0682	M	No
	187-50-6731	M	Yes
	271-60-0300	F	Yes
	212-72-1497	M	Yes
Group III	166-54-0441	F	Yes
Ribavirin	417-06-7388	M	Yes
only	567-55-4147	M	Yes
	395-87-4736	M	Yes

Subjects in groups I and II were inoculated i.v. with 0.5 ml of 10^{-1} dilution of Brownell plasma, previously shown to contain SFF virus. Group III subjects were inoculated with 0.5 ml of sterile saline.

Group I was given placebo capsules, while group II subjects received four (4) ribavirin capsules containing 100 mg of ribavirin at 08:00, 16:00, and 24:00 hours, beginning 1 day prior to virus inoculation and continuing through day 6. Group III received one additional dose (as above) of ribavirin capsules following day 6.

Ribavirin (virazole, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), Lot No. 30227, was formulated as 100-mg capsules by ICN Canada Limited for Viratek Inc., Costa Mesa, California. These capsules had an expiration date of 88-MR. Placebo capsules (Lot No. 40088) were also manufactured by ICN Canada Limited for Viratek. Both drug and placebo capsules were contributed by Viratek, Inc.

Endpoints for parameters measured over time, for each subject, were analyzed by repeated measured analysis of variance (ANOVA) after transformation to stabilize variances, or to normalize data as required. Tests for treatment differences, time trends, and treatment X time interactions were made by using appropriate error terms from the ANOVA. Treatment comparisons were also made within time by using estimates of the population marginal means and confidence intervals from the ANOVA. These tests have sufficient power since they use the within-subject error from the ANOVA (which has a large number of degrees of freedom). Power calculations were made for selected endpoints to assess the sensitivity of the experiment. Overall parameters measuring treatment impact (such as peak, time to peak, and total fever hours) were estimated. These parameters were tested by a one-way ANOVA for treatment differences. A multivariate ANOVA was run against treatment as the independent variable and partial correlations among the endpoints were examined to assess degree of association. All tests of hypothesis were conducted with alpha equal to 0.05.

Prior to statistical analyses, one placebo (459-37-7786) and two ribavirin (187-50-6731 and 161-48-0682) subjects were excluded due to failure to sero-convert. All efficacy findings were based on the remaining nine subjects (five placebo- in group I and 4 drug-treated in group II). Treatment effects were tested as described above and were considered statistically significant at the conventional $p<0.05$ level. Prior to testing for treatment effects, the significance of the immunization status for Rift Valley fever (RVF) was assessed by a 2 X 2 factorial analysis, crossing treatments (ribavirin or placebo) with immunization status. The interaction of immunization status with treatment was tested for statistical significance to enable us to decide if the analysis could proceed by ignoring prior immunization status. Sporadic and infrequent interactions were detected between prior immunization status and treatment groups (ribavirin and placebo) over time. Sample sizes were small at most time points, yielding low power for this test. The decision was made to pool immunization status groups for further analysis. A separate analysis of the pharmacokinetic group of four subjects was conducted. In this analysis, the variables were adjusted for baseline (day-1 for all variables), and the differences at each time point were analyzed for treatment effects between the ribavirin, placebo, and the pharmacokinetics groups. In this analysis, drug-related effects alone and in combination with the virus can be compared. The data means and trends were displayed by graphical displays and tables to illustrate the findings and conclusions.

Groups I and II

Placebo-treated subjects (group I) became febrile approximately 3.5 days after virus inoculation. Fever persisted for about 60 h, reaching a maximum of 102°F in four of the five placebo-treated subjects who sero-converted. In contrast, all ribavirin-treated subjects exhibited normal body temperatures and a normal diurnal temperature cycle throughout the hospitalization period.

Other vital signs consisting of diastolic and systolic blood pressure, pulse and respiratory rates, and body weight were unremarkable; no differences were observed between the placebo- and ribavirin-treated subjects.

Urinalysis findings were unremarkable. There were no effects observed which could be attributed to ribavirin treatment. There were no statistically significant differences observed for two consecutive or more days between placebo- and ribavirin-treated groups for any of the following clinical chemistries:

Albumin	SGOT	Na^+	Cl^-
Total protein	SGPT	K^+	Direct bilirubin
Glucose	LDH	Haptoglobin	Fe^{++}
Urea nitrogen	CK	C_3	IBC
Creatine	Cholesterol	C_4	Transferrin
Phosphorus	Triglycerides	IgG	C-reactive protein
Ca^{++}	HBDH	IgM	A 1 acid glycoprotein
Alk. phosphatase	T		

Total bilirubin was the only clinical chemistry parameter which was elevated significantly for two consecutive days. Values of 1.14 and 1.19 were obtained for ribavirin-treated subjects on days 5 and 6, compared to 0.46 and 0.41 for the placebo group ($p<.009$ and .006, respectively).

Sandfly fever antigen was detected by ELISA for 1-3 days in ill subjects. Sandfly fever virus IgM (ELISA) appeared on day 7, and IgG on day 9 in ill patients. Seroconversion occurred between days 12 and 28 in four of six ribavirin and in one of two asymptomatic placebo recipients. Serum interferon levels were elevated (1000 international units) only in ill subjects.

Hematological changes seen in the placebo group were consistent with a typical response to viral infections. These changes included a drop in granulocyte absolute counts from 3.62 on day 3 to 1.12 on day 8, then a rise to 2.60 by day 12. Statistically significant differences between the placebo and ribavirin groups were found on days 7-11. The relative percentage of granulocytes also dropped in the placebo group, from a value of 70 per cent on day 4 to 35 per cent on day 8. While there were no statistically significant differences between the placebo- and ribavirin-treated groups with regard to absolute lymphocytes counts, the percentage of lymphocytes increased significantly in the placebo group during days 6-9. Total WBC counts decreased significantly compared to those in the ribavirin-treated group on days 7-11, with a nadir of 3.3 ± 0.64 on day 8 compared to 7.60 ± 0.52 for the ribavirin-treated group.

None of the red cell indices, including red cell numbers, hematocrit, and hemoglobin, differed significantly between placebo- and ribavirin-treated groups. All three of these indices, however, showed a trend to lower values from the time of admission until day 10 of the study. In the ribavirn-treated group, reticulocytosis did develop, which was statistically significant from the placebo group on day 5-9 and 11-12. Peak reticulocyte counts reached a value of 4.62 ± 0.76 on day 11.

A consistent change in platelet values was observed between drug- and placebo-treated groups. Platelet counts in the placebo group decreased during the febrile period, from values above 200,000, to approximately 160,000 on days five to eight. On the other hand, platelet values in the ribavirin group increased, reaching a peak of 337,000 on day 11. The differences in platelet counts between groups I and II attained statistical significance on days 5-12.

Group III (Pharmacokinetics)

No clinical signs changes with regard to body temperature, systolic and diastolic blood pressure, and pulse and respiratory rates were noted in this group. Microscopic and laboratory urinalysis results were unremarkable throughout the study. All clinical chemistry values were within normal limits throughout the study, with the exception of direct bilrubin and serum iron.

Group III (drug control) experienced changes in hematological parameters analogous to those seen in the sandfly fever virus-inoculated/drug-treated subjects (group II). However, decreases in hematocrit, hemoglobin, and red blood cells were more precipitous than those seen in either group I or II. The magnitude of the observed changes from day of admission to day 8 of the study were decreases of 6.8 and 2.4 per cent, and 780,000 in the absolute values of hematocrit, hemoglobin, and red blood cells, respectively. These changes were occompanied by a reticulocytosis which reached a maximum of 4.23 on day 9. All values returned to normal limits by day 28. There were no other clinically or statistically significant changes in any other hemotological parameters.

Of special interest in this group were changes which occurred in hematocrit. Statistically significant drops from baseline were detected, starting on day 2 and continuing through day 28. However, statistically significant drops also occurred in the placebo group for days 4-5 and 7 and again on day 28, although the magnitudes of the drops were smaller than in the pharmacokinetics group. The ribavirin group showed similar drops as the pharmacokinetics groups in the same time periods. Thus, although the drops were larger in the drug groups, they were confined to the drug groups solely.

Plasma ribavirin levels were measured in the four subjects for the first four days of ribavirin administration and in three subjects thereafter. A plateau of about 6 μmoles was achieved by 96 h, with a maximum peak of 9.9 μmoles 2 h after dosing on day 4. Clearance of ribavirin was relatively slow, with serum levels of about 3.3 μmoles remaining 72 h after administration of the final dose.

Bone marrow aspirate and biopsies were obtained from two, group II subjects which had been treated with ribavirin for four and eight days, respectively. Samples revealed normocellular bone marrows. The biopsy showed the marrow to contain normal cellularity with adequate mitotic activity and an equitable distribution of erythroid and myeloid colonies. Bone marrow smears demonstrated the presence of all levels of erythro- and myelopoietic maturation. A low ME ratio was also noted, which probably reflects stimulation of erythropoiesis as a consequence of anemia. Whether these changes were in response to the 300 and 500 ml of blood drawn from these subjects at the time of the biopsy is not known.

In conclusion, ribavirin prevented clinical disease in subjects inocculated with sandfly fever virus, but did not prevent an immunological response to sandfly fever virus infection. This demonstration of ribavirin's antiviral activity against human sandfly fever has broader implications than simply the treatment of this high morbidity, but self-limiting, disease. As already demonstrated in experimental animals, ribavirin may also be active in man against several related viruses of this family, such as Rift Valley fever and Congo Crimean viruses. The fact that ribavirin has been shown to be clinically effective against influenza and respiratory syncytial virus signals the possibility that ribavirin may be used as a broad-spectrum antiviral agent to insure against the threat of virus outbreaks during military operations.

PRESENTATIONS: None

PUBLICATIONS: None

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3. **Canonico, P. G.** 1983. Ribavirin: a review of efficacy, toxicity and mechanism of antiviral activity, pp. 161-186. *In* F.E. Hahn (ed), Antibiotics, Vol. 6. Springer-Verlag, NY.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 305652	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	64758	3S464758D847	AN	002
b. CONTRIBUTING				
c. CONTRIBUTING	Cards			

11. TITLE (Precede with Security Classification Code) (U) Vaccine, Advanced Development

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	1.0	7.2
c. TYPE	d. AMOUNT	86	1.0	350
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Medical Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Cosgriff, T M
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2997
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Meadors, G F
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Vaccines; (U) Volunteers; (U) Phase III Clinical Trials; RAD I

23. TECHNICAL OBJECTIVE 24 APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Conduct field trials (phase III clinical testing) of experimental vaccines for prophylaxis of diseases of unique military importance, particularly those with potential as biological warfare threats.

24. (U) Experimental vaccines which have completed Phase I and II safety and efficacy testing will be studies in rigorously conducted field trials after extensive scientific and ethical reviews.

25. (U) 8410-8509-Preclinical vaccine studies conducted at USAMRIID in recent years have resulted in the development of a number of candidate vaccines against infectious and toxic agents. Some of these are still in preclinical testing. Others are in the initial phases of clinical testing. During the last years a joint protocol was developed with the British for a field trial of Rift Valley fever vaccine in British soldiers. British administrative difficulties have delayed the initiation of the trial. A field trial of the Junin vaccine is scheduled to begin shortly in Argentina within the endemic area for Argentine hemorrhagic fever. Initial testing of the vaccine will be carried out at USAMRIID.

BODY OF REPORT

PROJECT NO. 3S464758D847: Medical Defense Against Diseases of BW Importance

WORK UNIT NO. 847-AN-002: Vaccine, Advanced Development

PRINCIPAL INVESTIGATOR: Thomas M. Cosgriff, M.D., LTC

ASSOCIATE INVESTIGATOR: Gilcin F. Meadors, M.D., MAJ

Background:

One of USAMRIID's principal tasks is the development of effective vaccines against infectious and toxic agents which constitute a risk for military personnel. Once a candidate vaccine has been successfully developed, and preclinical testing demonstrates that it is safe and immunogenic and/or effective in animals, clinical testing is initiated. After Phase I and II clinical studies establish that the candidate vaccine is safe and immunogenic in humans, field studies of the vaccine involving large numbers of volunteers are conducted. These field studies, which are the subject of this report, not only provide additional evidence related to safety and immunogenicity, but also may provide direct evidence of efficacy, if the studies are carried out in areas where the biologic agent is endemic.

Summary:

The extensive preclinical vaccine studies which have been conducted at USAMRIID in recent years have resulted in the development of several promising candidate vaccines against viral, bacterial, rickettsial, and toxic agents. Some of these candidate vaccines are nearing completion of the preclinical phase of testing.

Others are in the intitial phases of clinical testing. During the last year, a joint protocol was developed with the British for a field trial of Rift Valley Fever vaccine in British soldiers. This protocol has not been initiated because of apparent administrative difficulties on the British side. It is uncertain whether this vaccine trial will ever be carried out. At this time, a study of a candidate Junin vaccine is being planned. This study is being conducted in Argentina within the endemic area of disease.

Progress:

While progress has been made in the ability of the Division of Medicine to organize and conduct clinical studies of candidate vaccines, support for field trials, given their large scope, must be provided by other elements of the Medical Research and Development Command, and the Army as a whole, to provide sufficient resources and a sufficiently large pool of volunteers.

Progress continues to be made in coordinating efforts with investigators in other parts of the world who have a common interest in developing a particular vaccine. This productive effort should prove to be of great benefit, both to the U.S. Armed Forces and to the populations of the countries involved.

PUBLICATIONS: None

PRESENTATIONS: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 308927	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
85 05 29	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	64758	3S464758D847	BA	003
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code) (U) Ribavirin

12. SUBJECT AREAS 0613 Microbiology; 1503 Defense; 0601 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
85 04	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	0.2	387
86	0.2	275

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Canonico, P G
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2290
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Huggins, J W
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Antiviral; (U) Ribavirin; (U) Chemotherapy; (U) Junin Virus; (U) Hantaan Virus; (U) Hemorrhagic Fever with Renal Syndrome; (U) RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop the drug ribavirin as an antiviral for the treatment of viral diseases of military importance.

24. (U) Conduct Phase III clinical trials appropriate for the development and FDA approval of ribavirin for the prophylaxis or treatment of serious viral infections. Establish liaison with medical authorities in appropriate areas to study the diseases. Design clinical trials and obtain appropriate U S and host country study clearances for conducting human trials. Conduct clinical trials to evaluate efficacy and toxicity in accordance with regulatory requirements.

25. (U) A clinical trial of the efficacy of ribavirin in treating clinically ill patients with the Chinese variant of hemorrhagic fever with renal syndrome, epidemic hemorrhagic fever, has been finalized with Hubei Medical College, Wuhan, People's Republic of China. All required approvals have been arranged and treatment will commence during the fall 1985 epidemic season. Up to 200 patients will be treated in order to evaluate drug efficacy. A clinical protocol for the evaluation of ribavirin against Junin virus, the causative agent of Argentine hemorrhagic fever, has been approved by the FDA and will be initiated during this fiscal year's epidemic season.

BODY OF REPORT

PROJECT NO. 3S464758D847: Medical Defense Against Diseases of BW Importance

WORK UNIT NO. 847-BA-003: Ribavirin

PRINCIPAL INVESTIGATOR: Peter G. Canonico, Ph.D.

ASSOCIATE INVESTIGATOR: John W. Huggins, Ph.D.

Background:

Ribavirin (1-β-D-ribofuranosyl-1,2,3-triazole-3-carboxamide), a nucleoside analogue with a close structural resemblance to guanosine, has been found to inhibit significantly a broad spectrum of both DNA and RNA viruses (1,2).

In randomized controlled studies, ribavirin, in a small-particle aerosol, has been shown to be effective in the treatment of type A and B influenza virus infections. Treated patients experienced more rapid defervescence, disappearance of systemic illness, and reduction of viral shedding than patients treated with placebo.

Ribavirin is the only antiviral agent which has been shown to have efficacy in the therapy of respiratory syncytial virus (RSV) infection. Other clinical studies have shown that ribavirin is effective in the treatment of Lassa fever in man (3). In these studies, patients with an admission viremia of $\geq 10^{5.6}$ tissue culture infectious doses (TCID) per ml had a $\geq$ 73% case fatality. Treatment with intravenous ribavirin within the first six days of illness reduced the mortality rate to 8% compared to 43% in those treated after day 6.

This work unit has expanded these clinical trials by examining the efficacy of ribavirin in two other militarily relevant viral diseases, hemorrhagic fever with renal syndrome (HFRS) and Argentine hemorrhagic fever (AHF).

HFRS is a collective term used to describe infectious febrile disease with hemorrhagic and renal manifestations. These disorders share the clinical "triad" of fever, hemorrhage (mild petechiae to severe ecchymosis), and renal damage (proteinuria, oligouria, uremia) in five overlapping phases: febrile, hypotensive, oligouric, polyuric, and recovery. Serologic and biochemical studies have established the relation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, with agents responsible for geographically diverse but clinically similar human diseases, including epidemic hemorrhagic fever (EHF) in Japan, China, and Eastern Europe. At present, tens to hundreds of thousands of cases are recognized annually in endemic regions (3).

Argentine hemorrhagic fever is a debilitating and often fatal disease of humans caused by Junin virus. In man, a spectrum of clinical illness is seen that ranges from a mild flu-like affliction to fulminant disease and death in 15 to 30% of untreated individuals (4). Typically, a 7- to 14-day incubation period is followed by fever, headache, loss of appetite, muscle aches, back and abdominal pain, dizziness, constipation, and diarrhea of about 1-week duration.

The disease exists in nature almost exclusively in the rich farmland provinces near Buenos Aires, Argentina. In most years, 200 to 400 cases are observed within the endemic region. However, for reasons which remain obscure, periodic large epidemics occur, involving thousands of individuals in this region. Moreover, a progressive extension of the endemic area (to ten times its 1958 size by 1980) has been documented (J. Maiztegui, personal communication). Consequently, the disease has had considerable impact on the welfare and economy of Argentina.

Summary:

A clinical trial of the efficacy of ribavirin in treating clinically ill patients with the Chinese variant of hemorrhagic fever with renal syndrome, EHF, has been finalized with Hubei Medical College, Wuhan, People's Republic of China. All required approvals have been arranged and treatment will commence during the fall 1985 epidemic season. Up to 200 patients will be treated in order to evaluate drug efficacy. A clinical protocol for the evaluation of ribavirin against Junin virus has been approved by the Food and Drug Administration and will be initiated during this year's epidemic season.

Progress:

The proposed field trial in China will be a double-blind, placebo-controlled trial of ribavirin for the treatment of EHF. Treatment will be initiated within four days after onset of earliest clinical symptoms in up to 200 patients. Two study sites will be utilized. One is the second attached hospital of the Hubei Medical College (HMC), that sees primarily urban cases of the disease, while the other will be at the Zong Chang County Hospital, that sees over 800 cases annually of the rural form of the disease. The infectious disease service of HMC will provide physicians experienced in the treatment of the disease as well as experienced in the conduct of clinical trials (a trial of interferon efficacy against EHF is currently being conducted at HMC for Dr. Monto Ho, University of Pittsburgh). The clinical investigators involved in that trial will also be involved in these trials.

Up to 80 volunteers will be included in the proposed field trial against Argentine hemorrhagic fever. All volunteers will be obtained from the patient population of the Instituto Nacional de Estudios sobre Virosis Hemorragicas, Pergamino, Province de Buenos Aires, Argentina. Prior to acceptance into the study, each volunteer will have culture or serologic testing to confirm that his illness is Argentine hemorrhagic fever, with symptoms and/or signs of disease in existence for more than eight days. Individuals will receive ribavirin or placebo, via a blinded-study design. Participants in the study will receive a complete physical examination at the time of admission, together with baseline virologic, serologic, hematologic, and biochemical studies. Each patient will be blood-typed and arrangements made for immediate cross matching with prospective donor unit(s) in the event of anemia (necessitating blood transfusion related to disease or ribavirin toxicity). Serial physical observations in concert with blood studies will be performed daily, or more frequently as clinically indicated. Volunteers will remain hospitalized for at least the duration of ribavirin therapy, and will be followed on an inpatient basis daily, or outpatient basis at least every other day, for two additional weeks.

PRESENTATIONS: None

PUBLICATIONS: None

LITERATURE CITED

1. **Canonico, P. G.** 1983. Ribavirin: a review of efficacy, toxicity, and mechanism of antiviral activity, pp. 161-186. *In* F.E. Hahn (ed.), Antibiotics, Vol. 6. Springer-Verlag, NY.

2. **Kirsi, J. J., J. A. North, P. A. McKernan, B. K. Murray, P. G. Canonico, J. W. Huggins, P. C. Srivastava, and R. K. Robins.** 1983. Broad-spectrum antiviral activity of 2-β-D-ribofuranoselenazole-4-carboxamide, a new antiviral agent. *Antimicrob. Agents Chemother.* 24:353-361.

3 **McKee, K. T., C. J. Peters, R. V. Craven, and D. B. Francy.** 1984. Other viral hemorrhagic fevers and Colorado tick fever, pp 649-677. *In* R.B. Belshe (ed.), Textbook of human virology. PSG Publishing Company, Inc. Littletown, Mass.

4. **Maiztegui, J. I.** 1975. Clinical and epidemiological patterns of Argentine hemorrhagic fever. *Bull. WHO.* 52:567-575.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 302626	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AP	131
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83 6.2/3			

11. TITLE (Precede with Security Classification Code) (U) Risk Assessment and Evaluation of Virus Agents and Their Vectors

12. SUBJECT AREAS
0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	2.5	142
c. TYPE	d. AMOUNT	86	1.0	140
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Bailey, C L
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2775
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Turell, M J
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Viral Diseases (U) Arthropod Transmission; (U) Entomology; (U) RAM I; (U) Lab Animals (U)Gerbils

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Identify arthropods and vertebrates associated with the maintenance and transmission of medically important arboviruses to man and define ecologic and environmental factors influencing the ability of arthropods to transmit viruses. Information will allow for development of methods for predicting relative risk of military personnel to arbovirus infections and for designing specific vector control strategies.

24. (U) Ecologic and environmental factors relating to an arthropod's ability to transmit viruses are studied under natural and controlled environments.

25. (U) Studies were conducted to compare the Rift Valley Fever RVF virus vector potential of *Aedes fowleri*, derived from specimens collected in Senegal, with *Ae. palpalis* collected from the Central African Republic. Both species were readily susceptible to oral infection. However, peak virus dissemination in *Aedes palpalis* was generally faster (14 days) as compared with *Aedes fowleri* (21 days). In another study, we discovered that vector mosquitoes that fed on gerbils with a concurrent RVF viremia and *Brugia malayi* microfilaremia developed disseminated infections at least 4 times more frequently than mosquitoes that fed on gerbils infected with RVF virus alone.

BODY OF REPORT

PROJECT NO. 3M162770A870: Medical Defense Against Infectious Diseases

WORK UNIT NO. 870-AP-131: Risk Assessment and Evaluation of Viral Agents and Their Vectors

PRINCIPAL INVESTIGATOR: Charles L. Bailey, LTC, Ph.D.

ASSOCIATE INVESTIGATOR: Michael J. Turell, Ph. D.

Background:

The recovery of Rift Valley fever (RVF) virus from adult *Aedes lineatopennis* reared from field-collected larvae in Kenya by Linthicum et al. (unpublished data), indicates the potential for vertical transmission to serve as a long-term maintenance mechanism for this virus. Laboratory studies are needed to evaluate factors which affect vertical transmission of this virus and to determine if stabilized infections of RVF virus can occur. While RVF virus has been isolated from numerous species of mosquitoes, additional vector competence studies are needed to determine the relative efficiency with which these, and potentially other, species can serve as vectors. The demonstration of mesenteronal escape and salivary gland infection barriers for arboviruses in mosquitoes by Kramer et al.(1) indicates the need to evaluate these barriers in potential vectors of RVF virus. Also, environmental factors (e.g., temperature of extrinsic incubation) and effects of arboviral infection on the potential vector need to be studied to determine how they influence the ability of mosquitoes to transmit that arbovirus.

Summary:

Two African species, *Aedes fowleri* and *Aedes palpalis*, were competent vectors of RVF virus following *per os* exposure. *Culex pipiens* with a disseminated RVF viral infection had reduced fecundity, refeeding rates, and survival as compared to uninfected individuals. Presence of uninfected larvae in a mosquito pool may inhibit recovery of virus from infected larvae. Temperature of extrinsic incubation was positively correlated with both infection and dissemination rates in *C. pipiens*, but only with dissemination rates in *Ae. taeniorhynchus*. Blood-meals consisting of an artificial viremia (defibrinated hamster blood plus previously frozen fetal rhesus lung-passed virus) were less efficient at infecting mosquitoes than were blood-meals consisting of defibrinated natural viremic hamster blood or artifical viremias containing unfrozen mosquito-passed RVF virus. Significantly more *Ae. aegypti* that ingested dengue-2 virus and microfilariae of *Brugia malayi* developed disseminated dengue infections than did those that ingested dengue-2 virus alone.

Progress:

A colony of *Ae. fowleri* (F_2-F_4) derived from specimens collected in the vicinity of Kedougou, Senegal, in October, 1983, and *Ae. palpalis* hatched from eggs obtained from females collected in the Central African Republic (C.A.R.), were evaluated for their ability to transmit RVF virus. Two strains of RVF virus were used: ZH-501 (obtained from a fatal human case during the Egyptian outbreak in 1978) and Dak ArB-1976 (=Zinga virus, obtained from a pool of *Mansonia africana* in the C.A.R.) Both strains had been passed in fetal rhesus lung cells prior to use in this study.

Both species were readily susceptible to oral infection with the Zinga strain of RVF virus (Tables 1 and 2). While virus disseminated rapidly in *Ae. palpalis* (no difference was observed in dissemination rates in mosquitoes assayed 7, 14, or more days after the infectious blood meal), peak viral dissemination did not occur until ca. 21 days extrinsic incubation with *Ae. fowleri*. In both species, about half of refeeding mosquitoes with disseminated infections transmitted RVF virus. The two strains of RVF virus were compared for their ability to replicate in, and be transmitted by *Ae. fowleri* following intrathoracic inoculation. Transmission rates were similar for the two strains, and no difference in transmission rates was observed by length of extrinsic incubation period (Table 3).

Our previous study demonstrated that mosquitoes that fed on gerbils with a concurrent RVF viremia and *B. malayi* microfilaremia developed disseminated infections at least four times more frequently than mosquitoes that fed on a gerbil infected with RVF virus alone (2). We replicated the basic study with a dengue-2 virus and *Ae. aegypti* model to determine if the increased vector capacity associated with the concurrent ingestion of virus and microfilariae was a general phenomenon. *Aedes aegypti* (F_2 from Bangkok, Thailand) were allowed to feed on either microfilaremic or amicrofilaremic gerbils. Each gerbil was inoculated either in the penis vein or both intracardially and in the penis vein with ca. 0.5 ml of an unfrozen dengue-2 suspension grown in *Ae. albopictus* (C_{6-36}) cells to create an artificial dengue-2 viremia. Only a low level viremia was obtained, ca. $10^{1.7}$ PFU of virus ingested per mosquito. While infection rates were similar in mosquitoes ingesting microfilariae and virus or virus alone, viral dissemination (as indicataed by the presence of virus in the legs) was fourfold higher in the mosquitoes feeding on the dually infected gerbil, as compared to the ones feeding on gerbils infected with dengue-2 virus alone (Table 4). Also, disseminated infections occurred earlier in the dually exposed mosquitoes, six days, as compared to 10 days for mosquitoes ingesting virus alone. Transmission of dengue-2 virus by bite was not attempted. The fourfold increase in viral dissemination confirmed our earlier study that the presence of microfilariae in a viremic blood meal enabled virus to bypass both the gut infection and midgut escape phases of the replication cycle.

Experiments were conducted to determine if the number of mosquitoes per pool affected recovery of RVF virus. While the inclusion of as many as 100 adult male or female mosquitoes per pool did not affect recovery of virus by plaque assay employing Vero cells, the presence of as few as five larvae in a pool significantly reduced the infectivity titer of RVF virus recovered. This phenomenon was observed for larvae of both species tested (*C. pipiens* and *Ae. aegypti*), and for pools "spiked" either by addition of an experimentally infected (inoculated) larva (Table 5), or by addition of a constant amount of RVF virus (Table 6). Inoculation of larval pools into hamsters detected virus in some pools that were negative by plaque assay, but failed to detect virus from some of the pools spiked with $10^{2.9}$ PFU/ml. An enzyme-linked immunosorbent assay (ELISA) was not affected by pool size, but failed to detect antigen from pools spiked with less than 10^3 PFU/ml. While inoculation of *Toxorhynchites amboinensis* was the most sensitive assay system tested for pools containing 10 or more larvae "spiked" by the addition of a viral suspension (RVF virus was recovered from pools that were negative by plaque assay, hamster inoculation, and ELISA), both ELISA and mosquito inoculation were equally efficient at detecting virus from pools containing an infected larva. These findings may need to be considered in the analysis of larvae for detection of transovarial transmission.

PRESENTATIONS: None.

PUBLICATIONS:

1. **Turell, M. J., C. L. Bailey, and C. A. Rossi.** 1984. Increased mosquito feeding on Rift Valley fever virus-infected lambs. *Am. J. Trop. Med. Hyg.* 33:1232-1238.

2. **Turell, M. J., P. A. Rossignol, A. Spielman, C. A. Rossi, and C. L. Bailey.** 1984. Enhanced arboviral transmission by mosquitoes that concurrently ingested microfilariae. *Science* 225:1039-1041.

3. **Turell, M. J., T. P. Gargan, II, and C. L. Bailey.** 1985. *Culex pipiens* (Diptera:Culicidae) morbidity and mortality associated with Rift Valley fever virus infection. *J. Med. Entomol.* 22:322-337.

4. **Turell, M. J., C. A. Rossi, and C. L. Bailey.** Effect of extrinsic incubation temperature on the ability of *Aedes taeniorhynchus* and *Culex pipiens* to transmit Rift Valley fever virus. *Am. J. Trop. Med. Hyg.* (In press).

5. **Rossignol, P. A., J. M. C. Ribeiro, M. Jungery, M. J. Turell, and C. L. Bailey.** Enhanced mosquito blood-finding success on parasitemic hosts: Evidence for vector-parasite mutualism. *Proc. Nat. Acad. Sci.* (In press).

LITERATURE CITED

1. **Kramer, L. D., J. L. Hardy, S. B. Presser, and E. J. Houk.** 1981. Dissemination barriers for western equine encephalomyelitis virus in *Culex tarsalis* infected after ingestion of low viral doses. *Am. J. Trop. Med. Hyg.* 30:190-197.

2. **Turell, M. J., P. A. Rossignol, A. Spielman, C. A. Rossi, and C. L. Bailey.** 1984. Enhanced arboviral transmission by mosquitoes that concurrently ingested microfilariae. *Science* 225:1039-1041.

Table 1. Vector competence of *Aedes fowleri* (F_{2-4}) for RVF Virus

	Dose Ingested				
Criterion	$10^{1.6}$	$10^{3.2}$	$10^{4.4}$	$10^{5.9}$	$10^{6.5}$
Inf. Rate[a]	13 (7/56)	58 (30/52)	79 (11/14)	73 (36/49)	92 (119/130)
Dis. Rate[b]	0 (0/22)	39 (13/33)	60 (6/10)	59 (17/29)	83 (52/ 63)
Dis./Inf (≤16[c]	0 (0/ 5)	20 (2/10)	0 (0/ 2)	31 (4/13)	41 (24/ 58)
Dis./Inf (≥18)[d]	0 (0/ 2)	65 (13/20)	67 (6/ 9)	74 (17/23)	87 (52/ 60)

[a] Percent infected (number infected/number tested).
[b] Percent with disseminated infection after 18 or more days extrinsic incubation (number with virus in less/number tested).
[c] Percent of infected mosquitoes with a disseminated infection for mosquitoes sampled after 5-16 days extrinsic incubation (number disseminated/number infected).
[d] Percent of infected mosquitoes with a disseminated infection for mosquitoes sampled 18 or more days after extrinsic incubation (number disseminated/number tested).

Table 2. Vector competence of *Aedes palpalis* for RVF virus (Zinga: Dak ArB-1976) following ingestion of viremic hamster blood

Criterion	Dose ingested				
	$10^{3.0}$	$10^{4.0-4.4}$	$10^{5.0-5.5}$	$10^{6.3}$	$10^{6.6-6.8}$
Inf. Rate[a]	36 (4/11)	62 (26/42)	73 (8/11)	77 (24/31)	89 (115/129)
Dis. Rate[b]	27 (3/11)	45 (19/42)	55 (6/11)	61 (19/31)	76 (98/129)
Trans. Rate[c]	0 (0/ 5)	33 (1/ 3)	0 (0/ 1)	50 (2/ 4)	45 (10/ 22)
Dis/Inf[d]	75 (3/ 4)	73 (19/26)	75 (6/ 8)	79 (19/24)	85 (98/115)

[a]Percent infected (number infected/number tested).
[b]Percent with disseminated infection (number with virus in legs/number tested).
[c]Percent transmitting by bite (number refeeding and transmitting/number refeeding).
[d]Percent of infected mosquitoes with a disseminated infection (number disseminated/number infected.

Table 3. Transmission of RVF virus by *Aedes fowleri* following intrathoracic inoculation

Days extrinsic incubation	Transmission rate (No. deaths/no. engorged)		
	Zinga[a]	ZH-501[b]	Total
7- 8	45 (8/18)	55 (6/11)	48 (14/29)
12-19	52 (11/21)	63 (5/ 8)	55 (16/29)
21-29	50 (9/18)	0 (0/ 1)	47 (9/19)
32+	33 (2/ 6)	not tested	33 (2/ 6)
Totals	48 (30/63)	55 (11/20)	49 (41/83)

[a]Dak ArB-1976
[b]Zagazig Hospital-501

Table 4. Effect of concurrent ingestion of microfilariae of *Brugia malayi* on vector competence of *Aedes aegypti* for dengue-2 virus

Days extrinsic incubation	Microfilaremic		Amicrofilaremic	
	Infection	Dissemination	Infection	Dissemination
6	15 (3/20)[a]	0 (0/20)[b]	20 (2/10)	10 (1/10)
8	25 (5/20)	0 (0/20)	15 (3/20)	5 (1/20)
10	26 (24/91)	4 (4/91)	23 (14/60)	15 (9/60)
Totals	24 (32/131)	3 (4/131)	21 (19/90)	12 (11/90)

[a]Number infected/number tested.
[b]Number disseminated (i.e., with virus in legs)/number tested.

Table 5. Comparison of various assay procedures for the detection of Rift Valley fever (RVF) virus from pools of *Culex pipiens* larvae "spiked" by the addition of 1 larva inoculated with RVF virus 24 h previously.[a]

Pool Size	Assay				
	Plaque[b]	Hamster[c]	Mosquito[d]	ELISA[e]	Any[f]
1	2.8(5/5)	4/4	3/4	3/4(3)	5/5(5)
5	1.5(4/5)	1/5	2/5	2/5(3)	4/5(4)
10	0.1(2/5)	1/5	2/5	2/5(5)	3/5(5)
25	<0.1(0/5)	1/5	5/5	5/5(5)	5/5(5)
50	<0.1(0/5)	0/5	4/5	2/5(4)	4/5(5)
75	<0.1(0/4)	2/4	0/4	2/4(2)	3/4(3)
Totals	11/29	9/28	16/28	16/28(22)	24/29(27)

[a]Each pool was triturated in 3 ml of mosquito diluent.
[b]Mean logarithm_{10} PFU/ml (number with plaques/number tested).
[c]Hamsters inoculated with 0.1 ml of undiluted suspension; number infected/ number tested.
[d]*Tx. amboinensis* inoculated with 1.7 μL of undiluted suspension; number infected/number tested.
[e]Number $\geq$ 3 standard deviations above background/number tested (Number $\geq$ 2 standard deviations above background).
[f]Number positive on at least 1 test - ELISA $\geq$ 3 standard deviations above background/number tested. (Number positive on at least 1 test - ELISA $\geq$ 2 standard deviations above background).

Table 6. Comparison of various assay procedures for the detection of Rift Valley fever virus from pools of *Culex pipiens* larvae "spiked" by the addition of $10^{2.9}$ PFU per ml.[a]

Pool Size	Assay method: Plaque assay[b]	Hamster[c]	Mosquito[d]
0	2.9(5/5)	5/5	5/5
1	2.2(5/5)	5/5	5/5
5	1.9(5/5)	2/5	4/5
10	1.2(5/5)	5/5	5/5
25	0.4(2/5)	3/5	5/5
50	<0.1(0/5)	4/4	4/4

[a]Each pool was triturated in 3 ml of mosquito diluent.
[b]Mean logarithm_{10} PFU/ml (number with plaques/number tested).
[c]+Hamsters inoculated with 0.1 ml of undiluted suspension; number infected/ number tested.
[d]*Tx. amboinensis* inoculated with 1.7 µL of undiluted suspension; number infected/number tested.

Table 7. Virus isolations from mosquitoes in Central African Republic - 1984

Genera	Total No. Pools	Total No. Mosquitoes Male/Female	No. Pools/ No. Mosquitoes With Virus
Culex	171	1625	-
Culiseta	9	16	-
Eretmapodites	20	79	-
Coquillettidia	13	35	-
Mansonia	17	117	-
Uranotaenia	14	36	-
Aedes	618	6672	3/75
Anopheles	22	105	-
TOTAL	884	8685	3/75

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 302630	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770	3M162770A871	AA	130
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Prevention and Treatment of Small Molecular Weight Toxins

12. SUBJECT AREAS 1503 Defense; 0613 Microbiology; 0605 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	1.0	304
86	0.0	0*

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Medical Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Bunner, D L
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7181
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Wannemacher, R W, Jr
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Neufeld, H A

22. KEYWORDS (Precede EACH with Security Classification Code) (U) T-2 Toxin; (U) Saxitoxin; (U) BW Defense; (U) Low Molecular Weight Toxins; (U) Military Medicine; (U) Rapid Detection; (U) Therapy

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop the ability to detect low molecular weight toxins in biological fluids, to study the basic mechanisms of action that relate to the development of potential therapy, and to develop prophylactic and/or postexposure therapy for soldiers exposed to these toxins. Emphasis is currently on trichothecene, certain marine, and blue-green algal toxins.

24. (U) Studies of basic chemistry (including structure), toxicology, distribution, metabolism, excretion, and pathophysiologic alterations will be made before developing therapeutic agents. HPLC and mass spectrometry are being used in addition to immunologic means of detecting agents and their metabolites in biological fluids.

25. (U) 8410-8509-Determination of the structure of a small-peptide, blue-green algal toxin has been made by mass spectrometry. Initial methods have been developed, using mass spectrometry, for saxitoxin. Improvement in immunoassays for trichothecenes, toxins, and some of their metabolites have also been accomplished; reagents are being generated for production of test kits. Charcoal, dexamethasone, and glutathione prodrugs, as well as soap and water decontamination, have all been shown to be quite successful for combatting trichothecene exposure. Anti-T-2 and anti-saxitoxin antibodies have been shown to be effective in vivo, even postexposure. Attempts to generate vaccines for testing are being undertaken.

*This research will be part of Work Unit 871AF in FY 86.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AA-130: Prevention and Treatment of Small Molecular Weight Toxins

PRINCIPAL INVESTIGATOR: David L. Bunner, COL

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Stephen R. Davio, Ph.D.
Donald A. Creasia, Ph.D.
Edward C. Hauer, M.S.

Background:

The low molecular weight toxin program has now been in progress for nearly five years, and the initial basic science information has gradually accumulated to the point that specific prevention and treatment are being developed. Although the majority of treatments are related to use of off-the-shelf drugs already tried in man, a significant portion of the treatment program has derived from more basic knowledge of the toxemias. Means of detection are available as well, with the emphasis on immunoassays.

Summary:

Mycotoxins Early testing to improve dosage pattern has been undertaken for several of the therapeutic agents known to be effective. Earlier drug development has slowed somewhat in favor of refinement of known effective agents. Detection of the trichothecenes has improved considerably, although an antibody for T-2 tetraol has not as yet been generated. New haptens have been injected into animals and await testing. Refinement of ELISA technology and determination of the efficacy of these assays in tissues has made significant progress. Absorption distribution studies are well in progress, documenting that dimethylsulfoxide (DMSO) markedly accelerates the absorption and that the barrier animal model is indeed a valid one. A more critical comparison of in vivo and in vitro data has demonstrated some contradictions. Toxins which are more potent in vivo may, in fact, be markedly less potent if assessed by protein synthesis inhibition alone. Some effort will be given to trying to determine the in vivo mechanism of action that might explain this discrepancy.

Saxitoxin In the saxitoxin family, the most significant progress has been in the development of effective, therapeutic antibodies. Immunological assays have been improved, although antibodies that also target the cogenors of saxitoxin are still needed. Some early attempts at potential vaccine generation have also been undertaken, and safety, as well as efficacy, will be addressed. A meeting on sodium channel effectors in the New York Academy of Sciences is scheduled for December and will be funded partially by the program.

Blue-green Algal Toxins The sequence of one of the microcystin toxins has been determined. The contract for generation of more of the blue-green algal toxins is in place, although this will likely take at least 6 to 12 months.

Progress:

Dermal Absorption Validation of in vivo absorption data was undertaken. Use of radiolabelled insulin, combined with a barrier, has shown that the barrier does in fact prevent absorption by other routes, such as oral. The toxicity of T-2 in methanol is some 50-fold reduced when a barrier is used. As to absorption in DMSO, however, the ultimate toxicity remains about the same, suggesting that an oral route is not significant with this vehicle after dermal exposure. Initial testing has suggested that pathogen-free animals are as much as 10 times more sensitive to the toxins. The mechanism of this is not known but will be pursued. A relationship has also been determined between the rate of absorption and the surface area exposed, suggesting that, even with the same amount of toxin, a greater total percentage is absorbed if a larger area is exposed. This is somewhat contradictory to in vitro data which also suggested that higher concentrations may increase the percent of absorption.

Cardiorespiratory Models A cardiovascular model in unanesthetized rats has been developed, and detailed information has been generated showing the classic response of hypotension, acidosis, alteration, and electrocardiographic changes; principally, prolongation of conduction and the sympathetic activation have been demonstrated. In saxitoxin poisoning, the most notable finding has been the recovery of animals after they reach apnea following injection of specific anti-saxitoxin antibody. The rapidity of this response is remarkable.

Analytic chemistry Much mass spectral data have been generated by using a number of probes with electron impact, chemical ionization, fast atom bombardment, and desorption chemical ionization. None has been wholly satisfactory, however. The greatest problem has been clean, easy sample preparation, particularly for T-2 tetraol. Two contractors, including Battelle Northwest and Northeastern University, are working in this area. A summer professor did initiate work on the mass spectral data for saxitoxin, with initial sensitivities in the range of 10 ng per injection. Also of note was the discovery that radiolabelled saxitoxin was quite sensitive, yielding multiple by-products. The peptides-sequencing of microcystin, a blue-green algal toxin (molecular weight: 994), has been determined.

Aerosol Models Aerosol studies are making continued progress. A brief assessment of particle size by use of various vehicles include powders at approximately 0.2μm, saline suspension .6μm), and ethanol solution at 1μm). The actual doses received have been determined by radiolabel, and the LC_{50} concentration in mice, young and old, using ethanol is 0.5 mg/kg and 1.55 mg/kg, respectively. In saline, the lethal concentration in younger mice is 0.3 mg/kg. Rat studies have only been initiated, but an LC_{100} at 250μg/L will ultimately result in a toxicity figure greater than from parenteral administration. The time to death is more rapid with certain of the aerosol doses and is suggestive of direct pulmonary effects rather than strictly systemic effects.

Computer Work A significant use of the SAS/graph for the division has been in progress and high quality posters with very short turn-around time have become common place. Three-dimensional, multi-variant methods for graphic representation have also been of assistance. The customization of data input and use of full screen mode on WYLBUR have also resulted. On-line access to CIS and BRS has been obtained and is being used at this time.

Immunology/Immunodiagnosis Specific work looking at the interaction of T-2 administration and *Candida* susceptibility has suggested that T-2 did, in fact, impair immunity, although final data are pending at this time. An ELISA methodology has been developed for T-2 with three different antisera: mouse monoclonal, rabbit polyclonal, and goat polyclonal. The monoclone can detect 100 ng/ml; the rabbit and goat sera are sensitive at 1 ng/ml. A saxitoxin ELISA has also been improved, with the sensitivity of 100 pg/ml which equals the membrane-binding assay that is several orders of magnitude better than the mouse bioassay. Detection of T-2 by immunologic methods in tissues has also been undertaken and appears to be comparably sensitive to use of radioactive tracers. A more detailed approach has also been given to the statistics of false-negative rates, and Mr. Gibbs has been of major assistance in this regard. More precise determination of the probability that a positive sample will be positive or that a positive sample will appear negative at any given dose level has been determined. This is particularly helpful in dealing with unknown samples taken trying to assess the meaning of results by using small numbers of samples. Affinity purification of goat, anti T-2 antibody has also made considerable progress and should be ready for testing against monoclones and assays. Significant progress has been made in the drug development program (reviewed under another work unit). Efforts are also underway in-house and by contract to develop monoclones to saxitoxin and the saxitoxin cogenors. This is being done by way of a saxitoxin-protein conjugate. Hope is for these antibodies to relate to both diagnostic testing and therapy. The use of specific saxitoxin-protein conjugates could also conceivably be used as vaccines if they prove to be sufficiently safe.

Metabolism Detailed studies investigating the administration of tritiated T-2 by parenteral and dermal routes to guinea pigs has been undertaken. The measurements were followed over 28 days. Tissue metabolites as well as urinary metabolites were determined, with the principal urinary metabolites being T-2 tetraol, 3'hydroxy HT-2 and 4-diacetylneosolaniol (DAS). The parent T-2 toxin was not detected in plasma, urine, or bile in the parenteral studies. Radioactivity was still present in tissues and body fluids 28 days after exposure to the toxin. Urine rather than blood, and T-2 tetraol, rather than T-2, are clearly the samples and metabolites, respectively, of choice, for diagnostic testing. As regards the topical exposure, it has been documented that the dermal areas act as a reservoir for the toxin and that the rate of uptake is faster when DMSO is the delivery solvent. The elimination, however, after dermal exposure in methanol is slower. T-2 in either vehicle can be detected in plasma but can only be detected in urine and bile by using the methanol vehicle. Inhibition of mitochondrial protein synthesis has also been studied in greater detail and compared to other agents, such as chloramphenicol. Stability studies of T-2, HT-2, and T-2 tetraol in urine and blood over six months of various temperatures have been undertaken. T-2 was unstable at all temperatures, although least unstable at -70°C. Tetraol is stable in all matrices tested. T-2 was stable in urine, although not in whole blood, at any temperature. This emphasizes the need to focus on tetraol as the principal metabolite, urine as the principal biological fluid, and improved methods for stabilization in the absence of -70°C freezers.

PRESENTATIONS:

1. **Bunner, D. L.** Low molecular weight toxins. Presented, Biologic Warfare Course, USAMRIID, 16-17 January 1985.

2. **Bunner, D. L.** Diagnosis and treatment of low molecular weight toxin exposures. Presented, Medical Management of Chemical Casualties. 8 May and 11 September 1985.

3. **Bunner, D. L.** Review of progress and diagnosis and treatment of biologic warfare agents. Presented, Technical Panel IV Meeting. Chemical Defence Establishment, London. 29 April-3 May 1985.

4. **Bunner, D. L.** Diagnosis and treatment of trichothecene toxin exposure. Presented, Mycotoxin Focus Officer Group, Suffield, Canada, 23-24 September 1985.

5. **Creasia, D. A., D. Thurman, R. Wannemacher, Jr., and D. L. Bunner.** Pulmonary toxicology of T-2 mycotoxin. Presented, Society of Toxicology 24th Annual Meeting, San Diego, CA., March 18-22, 1985.

6. **Davio, S. R., and J. F. Hewetson.** The development of anti-saxitoxin antibodies in Balb/c mice; antigen preparation and antibody detection. Presented, Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, 21-26 April 1985.

7. **Davio, S. R., J. F. Hewetson, and J. E. Beheler.** Progress toward development of monoclonal antibodies to saxitoxin; antigen preparation and antibody detection. Presented, Third International Conference on Toxic Dinoflagellates, St. Andrews, New Brunswick, Canada, 9-12 June 1985.

8. **Hewetson, J. F.** Army Research Office Conference on Detection Methods for Biological Agents. Raleigh, NC, April 1984.

9. **Martin, D. G., D. A. Creasia, and J. W. Parker.** Effect of intratracheal T-2 mycotoxin on respiratory gas exchange in the rat. Presented, Society of Toxicology 24th Annual Meeting, San Diego, CA, 18-22 March, 1985.

10. **Missler, S. R.** HPLC-MS of trichothecene mycotoxins. Presented, VG Analytical Users Meeting, University of California, Riverside, CA, 24 May 1985.

11. **Missler, S. R.** Determination of trichothecene mycotoxins by HPLC-MS. Presented, Annual Meeting of the American Society for Mass Spectrometry, San Diego, CA, 27-31 May 1985.

12. **Missler, S. R.** HPLC-MS and MS-MS of trichothecene mycotoxins, Gordon Conference on Trichothecene Mycotoxins, New London, NH, 17-21 June 1985.

13. **Thurman, J. D., D. A. Creasia, J. L. Quance, and A. J. Johnson.** Adrenal necrosis caused by T-2 mycotoxicosis in female but not male mice. Presented, Federation of American Societies for Experimental Biology, Anaheim, CA., 15-19 April 1985.

14. **Wannemacher, R. W., D. L. Bunner, J. G. Paca, and R. E. Dinterman.** Dermal absorption of T-2 mycotoxin in guinea pigs. Presented, Society of Toxicology, San Diego, CA. 18-22 March 1985.

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2. **Cosgriff, T. M., D. L. Bunner, R. W. Wannemacher, Jr., L. A. Hodgson, and R. E. Dinterman.** 1984. Th hemostatic derangement produced by T-2 toxin in guinea pigs. *Toxicol. Appl. Pharmacol.* 76:454-463.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 302642	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	H. TERM	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AC	132
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) (U) Exploratory Research for Protection Against Anthrax and Other Bacterial and Rickettsial Diseases

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; (U) 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	85 10	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	6.0	1199
86	0.0	0

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Bacteriology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Leppla, S H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Friedlander, A M
MILITARY/CIVILIAN APPLICATION M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Welkos, S L

22. KEYWORDS (Precede EACH with Security Classification Code) (U) BW Defense; (U) Microbiology; (U) Anthrax; (U) Vaccines; (U) Genetics; (U) Toxins; (U) Lab Animals; (U) Mice; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Study anthrax and other recognized bacterial and rickettsial pathogens having acknowledged BW potential and requiring special containment. Discover and characterize virulence factors; develop and exploit animal infection models; and study pathological and physiological effects of infection so as to identify and test candidate vaccine components.

24. (U) Characterize protein and polysaccharide antigens of pathogens by using biochemical and immunochemical methods; develop animal infection models in which to assess resistance mechanisms and evaluate candidate vaccine component efficacy; examine morphological and physiological responses to infection or immunization; use modern genetic methods to identify and isolate protective antigenic peptide domains.

25. Inbred mouse strains were shown to fall into three statistically different groups according to the rate at which they succumbed to anthrax infection. Breeding studies in mice showed that resistance to infection by Sterne strain is dominant and is associated with a single gene or gene complex. Mouse macrophages, the only cells known which are acutely damaged by anthrax lethal toxin (PA + LF), were shown to have cell-surface receptors, and to internalize toxin by endocytosis. Rat, guinea pig, and mouse, but not human monocytes, were highly sensitive to PA + LF. A high molecular weight *B. anthracis* cell-surface protein was identified which is immunogenic during vaccination with Sterne spores. *B. anthracis* was found to produce an oxygen-labile hemolysin. DNA sequence analysis of the PA and LF genes was begun. The gene for edema factor (EF) was shown to be on plasmid pXO1.

Continued under DA302643.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AC-132: Exploratory Research for Protection Against Anthrax and Other Bacterial and Rickettsial Diseases

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John R. Lowe, Ph.D.
Gregory B. Knudson, Ph.D.

Background:

In every analysis of bacterial pathogens that are likely to be developed as biological warfare agents, *Bacillus anthracis* finds a prominent place. Anthrax is a significant BW threat because the spores of this gram-positive bacterium are easily prepared, highly stable, readily disseminated in aerosols, and infectious by the pulmonary route. Infections progress rapidly and with nonspecific symptoms. The virulence of *B. anthracis* is attributed to two well-characterized factors: a polyglutamic capsule and a three-component protein exotoxin. Two types of vaccine are available to protect against anthrax infection. Live attenuated strains, such as the Sterne strain, are in wide use to protect domestic livestock and wild animals. Chemical vaccines, such as the currently licensed, U.S. human vaccine, consist of culture supernatants of the Sterne strain and contain, principally, the protective antigen (PA) protein. Epidemiological evidence suggests that the human vaccine does provide protection. However, animal studies show that live vaccines may provide protection against a wider variety of challenge strains and may induce a longer-lasting immunity. Possible routes to improving the human anthrax vaccine include either (1) selection and testing of attenuated live stains, such as the Sterne type, for use in humans; or (2) enhancement of the immunogenicity of chemical vaccines through admixing of additional antigens or enhancement of immunogenicity by altering the mechanism of presentation. Research to be reported in this work unit seeks to characterize the virulence factors of *B. anthracis*, to identify bacterial and host interactions which lead to infection and pathogenesis, and to develop improved vaccines and therapies for this disease.

Summary:

The genetics of virulence of *B. anthracis* was greatly clarified with the discovery of a second plasmid, pXO2, which codes for production of the polyglutamic acid capsule. Preliminary studies showed that a transposon from *Streptococcus fecalis* is able to function in *B. anthracis*, where it may be useful in generating mutants. Continued analysis of inbred mouse strains has shown that 10 strains fall into three groups based on their times-to-death following challenge with virulent anthrax spores. The large differences in susceptibility of inbred mouse strains to infection with Sterne spores was shown by extensive breeding experiments to reflect the existence of a single dominant gene or gene complex which confers resistance to infection.

A number of primary and established cell lines were examined for susceptibility to the anthrax lethal toxin (PA + LF). The macrophage was the only cell acutely damaged. Radiolabeled toxin was used to show that macrophages have cell-surface receptors which bind and internalize toxin by endocytosis. Comparison of macrophages from 11 strains of inbred mice revealed a clear separation into two groups based on sensitivity to the lethal toxin. A genetic analysis showed that F1 progeny were sensitive to toxin, indicating that sensitivity is controlled by a dominant gene.

Immunochemical analysis has shown that the cell surface of *B. anthracis* bacteria contains an easily extracted, antigenic, 93-kilodalton (kd) protein which is recognized during immunization and infection. Separate studies demonstrated that *B. anthracis* produces a hemolysin similar to that produced by other gram-positive pathogens.

Cloning of the PA and LF genes has progressed to the point that DNA sequencing can be undertaken. Convincing evidence was obtained that the EF gene is also located on plasmid pX01. A set of protein fusions between PA and galactosidase was obtained which will help to define the important domains of PA.

Progress:

Important progress was achieved during FY85 in understanding the genetic basis of virulence in *B. anthracis*. Prior studies had identified a single, large plasmid, pX01 (formerly designated pBA1), which codes for the toxin proteins. It has now been shown by a MRDC contractor, C. B. Thorne (1), in collaboration with USAMRIID staff, and independently by Japanese researchers (2), that virulent *B. anthracis* strains contain a second large plasmid, pX02, which codes for synthesis of the polyglutamic acid capsule. It is now clear that the Sterne strain is non-capsulated because it lacks pX02. Previous concern that Sterne might revert to full virulence can now be dispelled, and use of Sterne as a human vaccine can be undertaken with greater confidence in its safety.

Continued analysis of the genetics of virulence in *B. anthracis* would be aided by development of powerful mutagenic methods like those used for gram-negative bacteria. In particular, it would be advantageous if transposons were available. Preliminary work now has shown that TN916, a conjugative streptococcal transposon, may function in *B. anthracis*. Filter matings of *S. fecalis* DS16C2 with *B. anthracis* VNR-1 yielded tetracycline-resistant *B. anthracis* colonies. These recipients were subsequently shown to be donors of the tetracycline resistance character in matings with a streptomycin-resistant Sterne strain. No specific mutants have been obtained, so it will still be necessary to prove that insertion of the transposon is random and therefore useful in producing mutations. Once this is established, it will be practical to search for specific mutations. Highest priority will be given to selection of mutants defective in synthesis of all aromatic amino acids (aro^-). *Salmonella typhimurium* aro^- mutants have been shown to be avirulent and are receiving intensive evaluation as live vaccine strains. It is predicted that aro^- Sterne mutants would have decreased virulence; such mutants may prove to be totally innocuous live vaccines, suitable for use in humans.

Study of the pathogenesis of *B. anthracis* has not progressed rapidly in recent years, in part due to the unavailability of convenient animal models. The USAMRIID FY84 annual report described initial work on an inbred mouse model of anthrax infection. This work has now been extended to include ten inbred strains of mice.

All of the inbred mouse strains were, like outbred mice, highly susceptible to infection by virulent *B. anthracis*, such as the Vollum 1B. LD_{50} values were 5 to 30 spores for s.c. inoculation (data not shown). The inbrad strains could, however, be distinguished by their times-to-death, which ranged from 3.1 to 8.9 days (Table 1). An additional new finding was that the virulence of spores depended upon the growth conditions used in their preparation. Thus, spores grown on NBY agar appeared to kill CBA/J mice more slowly while being no different in their potency toward A/J mice. This result points out another source of variability in challenge experiments. Until careful and thorough studies are made of the factors in spore preparation which control spore potency, the production of spores of consistent and high potency will remain difficult.

In contrast to the response to Vollum challenge, the inbred mouse strains were divided into distinctly resistant or susceptible groups when challenged with noncapsulated, toxigenic *B. anthracis* such as Sterne or VNR-1. The latter bacterial strain is a derivative of Vollum 1B that has been cured of the pX02 plasmid (2). The lethal dose for susceptible mice (A/J & DBA/2J) was 1-2 x 10^3 VNR-1 or Sterne spores, whereas the other, more resistant *B. anthracis* strains required doses of 10^6 or more spores for lethality.

The unique susceptibility of the A/J and DBA/2J mice to infection by the relatively avirulent Sterne vaccine strain shows that these mice must be genetically defective in a mechanism for controlling infection. The availability of highly susceptible and resistant mouse strains has made it possible to undertake genetic analysis to determine whether resistance is determined by a small number of genes. A/J mice were crossed with CBA/J animals and the F1 progeny were tested for susceptibility with a dose of 2 x 10^4 Sterne spores. All of the progeny were resistant to infection. Subsequently, the F1 progeny were backcrossed to the parental susceptible (A/J) and resistant (CBA/J) strains. As predicted, the progeny resulting from the backcross to CBA/J were uniformly resistant to Sterne infection, whereas half of the mice from the backcross to A/J succumbed to Sterne infection (Table 2).

These data clearly support the hypothesis that host resistance to Sterne infection is dominant and is associated with a single autosomal gene, or gene complex. Genetic analysis of susceptibility to Sterne strain is expected to be of practical value in vaccine studies. In particular, the A/J mouse will be useful in testing the virulence of Sterne strain variants which have been altered so as to further decrease their pathogenic potential.

We hope that the differences observed above in the response of mouse strains to infection can eventually be attributed to differences in particular host-defense mechanisms. Research toward this goal has continued during FY85 through extension of the significant discovery that mouse macrophages are uniquely sensitive to the anthrax lethal toxin (PA + LF). The mouse macrophage system has been used to accumulate addition evidence that the anthrax toxin enters cells by receptor-mediated endocytosis. In the experiment described in Table 3, evidence was obtained suggesting that the toxin passes through an intracellular compartment.

In this experiment, macrophages were pretreated with ammonium chloride and then exposed to PA + LF at 37°C. Cells were then washed and reincubated in the presence of ammonium chloride while treated with antibody to LF in the cold for 1 h. After a further wash, cells were treated with either low or neutral pH and then incubated at 37°C. It can be seen that acid treatment induced toxicity and that antibody could not rescue cells previously exposed to toxin in the presence of ammonium chloride. This type of evidence has been used in studies with diphtheria toxin to argue that toxin accumulates in a cytoplasmic vesicle called an endosome, where it is inaccessible to neutralization by antibody. Exposure to low pH is believed to cause direct penetration of toxin into and across cell membranes.

If the sensitivity of macrophages to the lethal toxin were an important determinant of virulence, it would follow that the susceptibility of macrophages from various species would correlate with the species sensitivity to infection. To test this hypothesis, peritoneal macrophages from mice, rats, and guinea pigs were compared, with the results shown in Table 4.

It was found that macrophages from all three species are susceptible to toxin, although rat cells seem somewhat less sensitive. There appeared to be a residual population of both rat and guinea pig cells which were resistant to toxin. This may reflect the heterogeneity of cells obtained from the peritoneal cavity. To extend this comparison to human cells, the readily available peripheral blood monocyte was used. Human and mouse monocytes were prepared by similar methods and tested for sensitivity to toxin after one day in culture. The small numbers of mouse moncytes available required that this assay be performed by trypan blue exclusion. We observed that mouse monocytes were as susceptible as mouse macrophages, but human monocytes were totally resistant, whether tested one or seven days after isolation.

Researchers have continued to seek to identify cell lines sensitive to the acute toxic effects of LF. Previous tests showed that none of six established cell lines examined were sensitive to LF. Four additional established lines, which also proved resistant, are a human myelocytic cell, mouse sarcoma, and human and rabbit endothelial cell lines. Mouse neutrophils and red blood cells were also resistant. Thus, the monocyte macrophage is the only cell line which has been shown to be susceptible to the acute toxic effects of LF. Since all these studies have been done in vitro, it will be important to determine whether macrophages in vivo are equally sensitive.

The analyses of mouse cells described above have been carried out by using various substrains of the C3H mouse. In view of the differences between inbred mouse strains reported above, we asked whether cells from other inbred mouse strains were as sensitive as those from the C3H mouse. Peritoneal exudate macrophages from a number of mouse strains were cultured and exposed to varying concentrations of LF, with PA present at 1 μg/ml. The strains were clearly separated into sensitive and resistant groups, sensitive cells being killed at 0.01 μg/ml LF, whereas resistant cells showed < 30% lysis at 1 μg/ml. The results are summarized in Table 5.

As discussed for susceptibility to infection, the existence of susceptible and resistant groups makes possible a genetic analysis. Initial breeding experiments have been performed and we have found that the F1 progeny of C57BL/6J and C3H/HeJ mice have macrophages which are sensitive to toxin. This indicates that sensitivity to toxin is controlled by an autosomal dominant gene.

Two hypotheses have been examined to explain the differences in sensitivity to toxin, as shown in Table 5. The possibility that resistant cells might lack receptors was tested by measuring binding of radio-iodinated PA with the methods described in a subsequent paragraph. No dramatic differences in binding were observed between A/J and C3H macrophages. The second hypothesis we considered is that these macrophages differ in the acidity of their intravesicular compartments.

Attempts have been made to measure pH in macrophage vesicles with fluorescent markers. However, technical difficulties remain and other methods of measuring acidification are being considered. Macrophages from A/J and C3H mice have been compared for sensitivity to a number of bacterial toxins and viruses, but few differences in sensitivity have been found.

Important information about the internalization of other bacterial toxins has been obtained by using radioiodinated proteins. Therefore, methods were examined for radio-labeling PA, while retaining its biological activity. Success was achieved by using the Bolton-Hunter reagent. Binding of labeled toxin to cells was measured in the presence and absence of non-radioactive PA. Analysis revealed that there are approximately 50,000 toxin-binding sites per cell, with an affinity, K_D, of approximately 10^{-8}M.

Research studies to identify potential improvements in the human anthrax chemical vaccine are conceptually grounded on the consistent finding that stronger protection in guinea pigs is produced by immunization with Sterne strain spores than with chemical vaccines. This suggests the involvement of cell-surface antigens or of unrecognized, excreted antigens. The apparent inability of plasmid-cured strains to induce protection implies that any unrecognized antigens produced by the Sterne strain must be coded by plasmid pXO1. During this fiscal year, research was begun to identify immunogenic, cell-surface, bacterial antigens. Sterne cells were grown in chemically-defined R medium, and surface proteins were extracted by treatment with 1% SDS, 50 mM dithiothreitol (pH 8.5) at room temperature. Extracts were electrophoresed on polyacrylamide gels and proteins were electrophoretically transferred to nitrocellulose membranes. Immobilized antigens were detected after incubation with antisera and peroxidase-labeled conjugates. Antiserum from Sterne vaccinated guinea pigs recognized a 93-kd, cell-surface protein that was not recognized by antisera from guinea pigs immunized with the Michigan Department of Public Health (MDPH) chemical vaccine. This protein, termed extractable antigen (EA), was shown to be distinct from exotoxin proteins by monoclonal and polyclonal sera. Subsequent studies demonstrated that EA can be extracted rather specifically with 2 M guanidine-HCl. This cell-surface protein has been found in *B. anthracis* strains lacking either plasmid, indicating that EA is not plasmid-coded. Further studies will test the efficacy of this major cell-surface antigen as a vaccine in guinea pigs.

Although the pathogenesis of *B. anthracis* is widely considered to be due to the three-component anthrax toxin, it is important to critically evaluate the contributions of other exoproteins. *B. anthracis* is generally considered to be nonhemolytic. However, in the past year, several investigators have noted that *B. anthracis* does appear hemolytic under certain conditions. Review of the literature showed that the hemolysins of many gram-positive pathogens are of a type designated oxygen-labile. These hemolysins have an essential sulfhydryl group and are also characterized by their sensitivity to inhibition by cholesterol. With this information, a reconsideration of the hemolytic activity of *B. anthracis* was undertaken. Blood agar plates were prepared by using sheep RBCs that had been washed carefully to remove serum and cholesterol. On such plates, all strains of *B. anthracis* produced distinct, clear hemolytic zones. Analysis of crude toxin preparations indicated that an oxygen-labile, cholesterol-sensitive hemolysin was present. This enzyme could be purified by using the same column chromatography techniques in routine use for toxin purification. Fractions free of PA, LF, and EF were obtained which had high hemolytic activity, but still contained several polypeptides. In future work, homogenous hemolysin protein will be sought in order to compare the physical properties of this enzyme with the previously characterized hemolysins from *B. cereus* and *B. thuringiensis*. Recognition that *B. anthracis* also contains an oxygen-labile hemolysin, and development of a convenient agar plate assay, makes it possible to undertake genetic analysis of the hemolysin and to anticipate the selection of hemolysin-negative variants for use in pathogenicity studies.

Development of improved chemical and live anthrax vaccines will be greatly facilitated through analysis of the cloned genes for the three toxin components. Research in this area continued during FY85. Especially useful has been the library of pXO1 DNA, previously constructed by Dr. Don Robertson (formerly a visiting scientist, currently a MRDC contractor). The five, LF-positive, recombinant plasmids from this library have now been mapped by restriction enzyme digestion, and two of these five have been shown to produce full-size LF protein. Furthermore, one recombinant has been demonstrated to produce biologically active LF through use of the macrophage assay described previously. Mapping of the recombinants has allowed subcloning into M13 vectors appropriate for DNA sequence analysis. DNA sequencing has recently been initiated. Analysis of the putative, EF-positive recombinants continues to be limited by the uncertain quality of the oligonucleotide probes and the antisera. The question of whether the pXO1 plasmid codes for EF has, however, finally been resolved. This became possible when Curtis Thorne, an MRDC contractor, developed methods for transfer of plasmids among *Bacillus* strains. Dr. Thorne provided *B. cereus* strains which contained the pXO1 plasmid, and these strains were analyzed for EF production by using both CHO cell elongation and adenylate cyclase assays. Both assays demonstrated EF production by the *B. cereus* containing pXO1 plasmid. Knowledge that pXO1 contains the EF gene makes it worthwhile to continue the analysis of the weakly immuno-reactive, putative, EF recombinants. It is possible that in construction of the library, clones containing the entire EF gene were lost because they produced active adenylate cyclase which perturbed the metabolism of the *E. coli* host. This possibility can be examined once sequence analysis of the putative EF clones is completed.

In the genetic analysis of toxin genes, the gene for PA has been most intensively studied, both because it was the first gene to be cloned and because PA occupies the central position in immunity to anthrax. The original recombinant plasmid, pSE36 was subcloned to reduce the insert size to 4 kilobases (kb), yielding plasmid pPA26. Several investigators obtained data suggesting that pPA26 might not produce full-size PA. However, during this reporting period, careful analysis confirmed that this 4-kb insert does code for biologically active protective antigen. Since it is critical that the DNA sequence of the protective antigen gene be obtained, arrangements have been made to have this sequence determined, under contract, by Meloy Laboratories.

In a second project with the cloned PA gene, the genetic protein fusion technique is being used to identify essential antigenic and functional domains. The 6-kb DNA fragment from pSE36 was inserted into the vector pMLB1034, which consists of a multilinker site adjacent to the *lac* Z gene of *E. coli*. The presence of the intact PA gene was confirmed by DNA hybridization and production of intact PA was demonstrated by ELISA and Western blot analyses. Deletion events were then selected which cause fusion of the PA and *lac* Z genes. Many such clones were isolated and analysed. Deletions were obtained that ranged from 0.2 to 5 kb (Table 6).

Continued analysis of these fusion proteins has been difficult due to the small amounts of protein produced. Affinity techniques are being explored to isolate the fusion protein from *E. coli* lysates. However, it may be necessary to insert the fused gene into expression systems in order to obtain amounts of proteins which are sufficient to analyze their protein structure. Further analysis of these fusion proteins in functional assays and through use of monoclonal antibodies should help to analyze the detailed structure of the PA protein.

Research on anthrax toxin would benefit greatly through identification of the enzymatic mechanism-of-action of LF. A finding which may provide an entry into this area and was reported in the FY84 Annual Report, was the demonstration that PA causes the incorporation of ^{32}P from gamma-labeled ATP into macrophage proteins. This study has now been resumed by a new National Research Council (NRC) associate (E. Cora); he has demonstrated that the apparent phosphorylation reaction does not require eukaryotic cell materials. Instead, he observed that phosphorylation occurs when crude anthrax toxin is incubated with ATP. An important new finding is that calcium ion greatly stimulates phosphorylation. Fractionation of the crude toxin will be performed to determine which component or impurity in the crude toxin is responsible for this activity.

Studies on another bacterial pathogen, *Bordetella bronchiseptica*, have been performed in collaboration with Animal Resources and Airborne Diseases Division personnel. Infection by this pathogen has been a recurrent problem in guinea pigs bred and housed at USAMRIID. Control of this infection had been achieved in the past by using a vaccine produced by the National Institutes of Health. Unavailability of this vaccine required testing of a commercial material, Bronchicine, produced by Dellin Laboratories. An ELISA test with Bronchicine vaccine as antigen demonstrated prior exposure of 10 to 30% of strain 13 guinea pigs to *B. bronchiseptica*. Vaccination trials were conducted with animals proven to be free of prior exposure. Animals were vaccinated with Bronchicine, shown to develop a mean titer of 500 after two weeks, and were then challenged with 100 LD_{50} by the aerosol route. Whereas all control animals died, vaccination with Bronchicine led to complete protection. It was concluded that Bronchicine is safe and efficacious. This material is now used routinely to vaccinate USAMRIID's strain 13 guinea pig colony.

PRESENTATIONS:

1. **Welkos, S., R. Rotella, and T. Keener.** 1985. Susceptibility of inbred mice to infection by *B. anthracis* and to anthrax toxin. Presented, Annual Meeting, American Society of Microbiology, 3-8 Mar 1985, Las Vegas, NV.

2. **Leppla, S.** 1985. Genetics of anthrax toxin. Presented, Second Annual Workshop on Bacterial Protein Toxins, 3 July 1985, Wepion, Belgium.

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1. **O'Brien, J., A. Friedlander, T. Dreier, J. Ezzell, and S. Leppla.** 1985. Effects of anthrax toxin components on human neutrophils. *Infect. Immun.* 47:306-310.

2. **Ezzell, J., P. Mikesell, B. E. Ivins, and S. H. Leppla.** 1985. The genetic basis of Pasteur's attenuation of *Bacillus anthracis* cultures, pp. 107-116. *In* H. Koprowski and S. A. Plotkin (eds.), World's Debt to Pasteur. The Wistar Symposium Series, Vol. 3. Alan R. Liss, N.Y.

3. **Leppla, S. H., B. E. Ivins, and J. W. Ezzell.** 1985. Anthrax toxin, pp. 63-66. *In* L. Leive (ed.), Microbiology 1985. American Society for Microbiology, Washington, D.C.

4. **Ezzell, J. W.** 1985. Anthrax. *In* C. Gyles and C. Thoen (ed.), Pathogenesis of bacterial diseases. Iowa State University Press, Ames, IA.

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Table 1. Differences in susceptibility of mice to lethal infection with virulent *B. anthracis* (Vollum 1B)

Susceptibility Group[a]	Mouse Strain	Time-to-Death (days)[b] Leighton-Doi Broth Spores[c]	NBY Agar Spores[c]
	DBA/2J	3.1	--
I	A/J	3.2	3.5
	C3H/HeN	3.5	--
	C57BL/6J	4.1	--
II	C57L/J	4.3	--
	C3H/HeJ	4.8	--
	C58/J	5.2	--
	CBA/J	5.4	8.8
III	C57BR/cdj	6.5	--
	BALB/cJ	8.9	--

[a] Mice were divided into three significantly different groups ($p=.002$) by multiple pairwise comparisons (Bonferroni method) of the regression coefficients for each mouse strain (data not shown). Comparisons were made at the $\log_{10}$ geometric mean dose (60 spores) of Vollum 1B and were made relative to C57BL/6J.
[b] Harmonic mean time-to-death of mice infected with the geometric mean dose.
[c] Spore inocula were prepared from cultures of *B. anthracis* (Vollum 1B) grown in either Leighton-Doi broth or on nutrient broth-yeast extract agar.

Table 2. Susceptibility of mice to Sterne: backcross analysis

Mice	Mortality No. Dead / Total No.	Observed (%)	Expected (%)[b]	X^2	p
F1 x A/J:[a]					
Females	15/29	51.7			
Males	15/34	44.1			
TOTAL	30/63	47.6	50	.115	N.S.
F1 x CBA/J:[a]					
Females	0/30	0			
Males	0/29	0			
TOTAL	0/59	0	0	0	N.S.

[a] F1 mice are the (A/J x CBA/J)F1 progeny. For both matings, the female parent is indicated on the left and the male on the right.
[b] Percentages expected for a trait controlled by a single dominant gene.

Table 3. Effect of Pretreatment with Antibody on Acid pH induced Toxicity[a]

Antibody	pH Treatment	LDH (mU/well)
No	Neutral	568.2 ± 32
No	Acid	78 ± 4.5
Yes	Neutral	565 ± 18.6
Yes	Acid	90.9 ± 6.4

[a] Adherent C3H mouse peritoneal exudate macrophages were incubated in media containing various agents, and PA and LF at 1 μg/ml. Viability was measured as the LDH activity remaining in the monolayer.

Table 4. Relative Susceptibility of Mouse, Rat, and Guinea Pig Macrophages to LF[a]

	Cellular LDH (% Control)		
LF (μg/ml)	Mouse	Rat (Fisher 344)	Guinea Pig (Hart)
0	100	116 ± 12	116 ± 8
0.0001	81 ± 2	115 ± 9	45 ± 4
0.001	6 ± 1	41 ± 2	29 ± 1
0.01	2 ± 1	15 ± 3	15 ± 2
0.1	1 ± 1	11 ± 1	23 ± 1

[a] Peritoneal macrophages from various species were cultured. PA was present in all wells at 1 μg/ml. LDH was measured after 24-h exposure to toxin.

Table 5. Susceptibility of Various Mouse Macrophages to LF

Sensitive	Resistant
C3H/HeNsd	A/J
C3H/HeJ	C57BL/6J
CBA/J	C57BL/10J
BALB/cJ	AKR/J
Swiss	DBA/1J
	DBA/2J

Table 6. DNA Deletions in Recombinant Lac$^+$ Clones

Deletions in Recombinant DNA		Representative Plasmids:	
Size Class	Kb	Approx. Size (Kb)[a]	Strain
--	--	None	2-1-20B (parent)[b]
1	>4.5	5.1	C4-3-A
2	4.0-4.5	4.4	1-2-A-Mac
3	3.5-4.0	3.9	2-1-20B-1
4	3.0-3.5	3.3	2-1-20B-6
5	2.0-2.5	2.5	2-1-20B-2
6	<1.0	0.25	2-1-20B-4
		0.21	2-1-20B-3
Deletion in vector (no insert)		0.6-1.0	pMLB-7B

[a]Size of deletion in the plasmid DNA insert containing the PA gene. Results are based on mapping by single and double restriction endonuclease digestions with Bam HI, Sma I, Eco RI, Pst I, Hinc II, and Pvu II.
[b]The *Lac*$^-$, PA-producing recombinant constructed by inserting the 6.0-kb, Bam HI-fragment of pSE36 into the Bam HI-site of pMLB1034.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 302643	85 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AD	133
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Research for Protection Against Arenaviruses

12. SUBJECT AREAS
0613 Microbiology; 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	2.0	518
c. TYPE	d. AMOUNT	86	10.2	2353
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19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Disease Assessment Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Jahrling, P B
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7244
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Kenyon, R H
MILITARY/CIVILIAN APPLICATION M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Liu, C T

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Vaccines; (U) Lab Animals; (U) Guinea Pigs; (U) Primates; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To isolate, study, and characterize agents of potential BW threat. To obtain immunogens that elicit protective immunity and to devise effective regimens to protect US military personnel in the field.

24. (U) Naturally occurring and laboratory-derived strains are molecularly and biologically characterized and assessed for virulence or attenuation in susceptible animal models. Attenuated strains are tested for ability to induce cross-reactions with virulent strains. Protective immunity elicited by inactivated antigens is determined.

25. (U) 8410 - 8509 - Lassa virus (LV) strains isolated from human patients in Liberia and Nigeria and inoculated into guinea pigs and cynomolgus monkeys exhibited a spectrum of virulence similar to human disease severity. Occasional isolates from lethally infected patients, especially pregnant women and infants, were totally benign for guinea pigs. Studies are underway with Junin virus and effects of interferon upon infection in guinea pigs. In anthrax studies, a high-molecular-weight, *B. anthracis*, cell-surface protein was immunogenic during vaccination with Sterne spores. DNA sequencing of PA and LF genes was begun. Genome segments of Hantaan virus were cloned and partially sequenced. Data suggest that the medium encodes the 2 viral envelope glycoproteins. High-mannose, N-linked carbohydrate residues on both envelop proteins are important to viral replication, Mouse peritoneal macrophages variously sensitive to *Coxiella burnetii* were evaluated for activation. Inactivated, phase I whole cells activated the peritoneal macrophages of 3 strains of mice, as determined by nonspecific tumoricidal activity and reduction in 5'-AMPase activities.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AD-133: Exploratory Research for Protection Against Arenaviruses

PRINCIPAL INVESTIGATOR: P. B. Jahrling, Ph.D.

ASSOCIATE INVESTIGATORS: R. H. Kenyon, Ph.D.
C. T. Liu, Ph.D

Background:

The arenavirus taxon includes four significant human pathogens (1). Lassa virus is the etiologic agent of Lassa fever (LF), a severe, often fatal disease now recognized to be endemic to regions of West Africa; tens of thousands of LF cases occur there annually. Junin and Machupo viruses are associated with Argentine and Bolivian hemorrhagic fevers (AHF and BHF, respectively). Several hundred to thousands of AHF cases are recognized annually; BHF is presently less of a public health problem, but its potential to cause devastating epidemics is well documented. Lymphocytic choriomeningitis virus (LCM) may have a worldwide distribution and causes significant morbidity but low mortality in human populations. All of these viruses have a demonstrated potential to produce explosive outbreaks under artificial conditions; all are highly infectious by aerosol. An understanding of the basic pathophysiological processes underlying these diseases and the protective immune responses necessary for recovery is prerequisite to the development of effective vaccines and therapeutic measures to protect and treat effectively at-risk populations. Animal models are being developed for these viruses as well as related, but less dangerous, arenaviruses, including Pichinde.

Summary:

An additional 66 Lassa viral strains were isolated from human patients in Liberia; these exhibited a spectrum of virulence for guinea pigs that usually reflected human disease severity, except for occasional isolates from fatally infected pregnant women and infants. Comparison of Junin viral strains that differed in virulence for human AHF patients was similarly reflected by the guinea pig model. Some strains were viscerotropic, while others spared extraneural tissues and eventually invaded the central nervous system (CNS). Tropism and virulence were related in part to the humoral immune responses and sensitivities of the strains to antibody and complement. Pathophysiological responses of Pichinde virus-infected guinea pigs suggested a major role for leukotrienes, resulting in decreased cardiac contractility, output, increased capillary permeability, pulmonary edema, and hypotension. Prostaglandins, myocardial depressant factor, catecholamines, ACTH, and cyclic AMP may also be involved in this lethal model arenavirus infection.

Progress:

Continued characterization of Lassa viral isolates from Liberian patients. In addition to the 63 Lassa viral isolates obtained last year from Liberian LF patients, an additional 66 isolates were obtained this year from the sera of 543 patients with febrile illnesses compatible with LF. These sera were obtained by Dr.

John Frame and colleagues under contract. Twenty-three of the 66 patients confirmed to have LF died; the 43 survivors were added to the 29 individuals who seroconverted without yielding virus; these constitute a new prospective donor pool for immune plasma. All isolates were identified presumptively as Lassa virus by immunofluorescence and confirmed by neutralization (2).

Virulence testing of these isolates and ELISA testing for antigen and antibody (2,3) were curtailed because of the renovation of the Institute's Lassa suite from August 1984 to March 1985, and the sabbatical leave of the principal investigator from January to December 1985. However, 30 isolates were virulence-tested in strain 13 guinea pigs during the reporting period; 9 were benign, and of these, 7 were isolated from pregnant women, and 2 from infants who died. These results, while still incomplete (viremias remain to be tested), extend the observation made last year that human virulence of Lassa virus is closely reflected by guinea pig lethality, but that guinea pig-benign strains are obtained occasionally from fatally infected patients. This stresses the importance of host factors in resistance to LF (Table 1).

<u>Studies with Junin virus virulence</u> Studies related to the basis of attenuation and virulence of Junin viral strains were continued. Low-passage-level isolates from human Argentine hemorrhagic fever patients exhibited a broad spectrum of illness in guinea pigs from acute visceral disease to strains exhibiting predominantly neurological manifestations (Table 2). The visceral form typically showed virus replication mainly in spleen, lymph nodes, and bone marrow by 5 to 6 days post-infection. At death (days 13 to 17), there were 5 to 6 logs PFU/g in these tissues. Fewer viruses were found generally in blood, and viral titers in brain tissue usually approximated that found in blood. At death, the predominant lesions were necrosis and cellular depletion of spleen, lymph nodes, and bone marrow. These were generally not severe enough to cause death. In contrast, with the neurological forms of the disease, low levels of virus were detected in spleen and lymph nodes by days 5 to 7, but by days 10 to 13, virus was no longer detectable in these animals. However, between days 20 and 30, virus was detected in brain tissue, and reached titers of ≥ 5 logs/g after day 25. Usually this was manifested by rear limb paralysis, which, over a 4 to 5 day period, became progressively more severe. Low levels of circulating antibody had often appeared in these animals by the onset of paralysis. Brain tissues from the paralyzed animals generally showed moderate polioencephalitis. Preliminary data with one of the strains showing a neurological pattern of illness showed severe lesions in the olfactory bulbs, suggesting that virus may enter the brain via the olfactory epithelium. Although most of our studies with the various strains were performed with Hartley strain outbred guinea pigs, we also inoculated inbred strains 13 and 2 guinea pigs with virulent, mild, and attenuated strains of Junin. We observed no increased susceptibility to the virus or no significant change in disease patterns.

In addition to the in vivo studies with the virulent and attenuated Junin strains, we examined in vitro differences of these viruses as well (Table 3). Any one of these differences by itself probably cannot account for virulence, but each may contribute to the overall phenotypic expression. Although all the data on these strains have not yet been gathered, a pattern for virulence/attenuation begins to emerge. Some strains were highly viscerotropic, rapidly grew in lymphatic tissue, and killed their host during this phase of illness. Others were less rapid growers, and eventually infected and grew in the brain of animals. Available data have yet to show a role for cell-mediated immunity in control of the disease; however, antibody seems to be the critical factor. Some strains seem to destroy almost completely antibody synthesizing mechanisms, whereas other strains seem to gain access to the brain where, due to the blood-brain barrier, they are less vulnerable to the low levels of circulating antibody. Another factor may be subtle differences in surface antigens expressed on virus particles and infected cells. Although the molecular differences have not yet been examined, the manifestation of these differences appear to be that attenuated viruses are more easily neutralized than virulent virus, and cells infected with attenuated virus are more easily lysed by antibody and complement than are cells infected with virulent virus.

Ribavirin

We examined the efficacy of ribavirin in treatment of guinea pigs infected with AHF (Table 4). Ribavirin significantly increased the mean time-to-death, both delayed and suppressed viral replication, effectively lowered organ viral titers, and eliminated or reduced viremia to undetectable levels, but did not enhance survival of these animals. The treated animals usually died with high-viral titers in the brain and frequently were paralyzed. Low levels of antibody often were detected in the animals at death, but most likely developed too late and/or were in too low titer to offer protection to the brain. The beneficial effects of ribavirin may be amplified in human AHF therapy, because humans are probably more resistant to the virus.

Pathophysiologic studies of Pichinde virus-infected strain 13 guinea pigs

Progressive cardiac depression develops during Pichinde viral infection in strain 13 guinea pigs. Significant decreases of cardiac output and stroke volume without marked changes in heart rates were previously shown on day 7 post-inoculation. In other experiments, coronary vasodilatation plus depressed cardiac contractility and decreased ventricular pressures were observed in the isolated, perfused, working hearts of virus-infected animals. Since virus was not detected and few histopathological changes were demonstrated in the failing heart, experiments were designed to study cardiac electrical activities by measuring the ECG (electrocardiogram) of the infected animals under anesthesia. ECG patterns changed little after infection except that the magnitude of P and R waves, and total voltages from the QRS complex vector analyses increased by day 10. Double P waves occasionally were seen. The enhanced amplitude of the ECG changes of infected guinea pigs was maintained until shortly before death. These findings suggest that, although the cardiac muscle was gradually weakened during Pichinde infection, the rate and pattern of activation of the cardiac conducting system were not adversely altered. Thus, therapeutic strategies under these conditions should be directed toward improvement of cardiac contractility, rather than attempting to manipulate the conduction system of the heart.

Leukotrienes and pathogenesis of Pichinde virus Infected guinea pigs demonstrated progressive decrements in cardiac function, developed pulmonary edema, and died within 14 days of inoculation. Since viral infection or histological changes could not be detected in the heart, we postulated that leukotrienes may induce cardiac dysfunction and increased vascular permeability. A potent leukotriene antagonist, FPL-55712 (4 and 10 $mg^{-1}kg^{-1}day$), was also injected s.c. into the virus-infected animals on post-inoculation days 3 through 21. The magnitude of body weight loss decreased, food intake increased, and survival time was prolonged by 7 days.

Under contract, Dr. Robert C. Murphy analyzed plasma leukotrienes by bioassay and radioimmunoassay kits (New England Nuclear, Boston, MA). Table 5 summarizes the results from determinations of plasma sulfidopeptide leukotrienes in control and Pichinde virus-infected guinea pigs. In the control group, although a trace amount of myocontractile substance(s), (equivalent to 3 ng of LTE_4 activity), was detected in plasma, radioimmunassay did not confirm the presence of immunoreactive leukotrienes. Similar negative results also were obtained on four of five plasma samples from guinea pigs on day 7 post-inoculation. However, one animal had 10- to 20-ng equivalents of LTE_4 by bioassay, but less than 1 ng LTC_4, in radioimmunassay. All five samples from day 11 were strongly reactive in the guinea pig ileum assay (10 ng to > 20 ng LTE_4 equivalents) and, furthermore, yielded values of 1.1 to 2.4 ng of LTE_4 equivalents in radioimmunoassay. Six day-14 samples were essentially negative in both assays and the seventh had myocontractile activity equivalent to 2.4 ng of LTE_4 (sulfidopeptide leukotrienes).

The positive identification of sulfidopeptide leukotrienes in plasma of infected guinea pigs on day 11 post-inoculation provided evidence that, during the course of Pichinde viral infection, a circulating mediator with adverse activities was probably released. It is not known whether the circulating leukotrienes are responsible for organ dysfunctions. Nevertheless, many recognized effects of leukotrienes are seen in the Pichinde-infected guinea pig: decrease in cardiac contractility, output, and cardiac alteration of coronary vascular resistance, increased capillary permeability, pulmonary edema, hypotension, and other clinical signs. Since FPL-55712 significantly prolonged survival by day 7 post-inoculation and there was some evidence that sulfidopeptide leukotrienes appeared in the plasma of infected guinea pigs on day 11, these combined facts strongly suggest that leukotrienes are partially involved in the critical pathogenetic process leading to eventual death.

Leukotrienes may not be the only chemical mediators occurring in the circulation during Pichinde viral infection. Other possible chemical compounds, including prostaglandins, myocardial depressant factors, catecholamines, adrenal cortical hormones, and cyclic AMP may also be responsible for pathogenesis of this viral disease. Although sufficient doses of FPL-55712 did not prevent death from the overwhelming Pichinde viral infection, leukotrienes may be involved in developing capillary leakage and a gradually induced cardiac depression without producing tissue damage or marked histological changes. If this hypothesis is true, arenavirus-infected patients may one day be managed with leukotriene antagonists to improve critical organ functions and intravenous infusion of colloid to combat hypovolemia, thus providing a window for further treatment with effective antiviral drugs, including ribavirin or a mixture of ribavirin and selenazole. If the arenavirus-infected patient's life can be prolonged beyond a certain critical point by these means, a specific immune response may contain the viral infection. Consequently, eventual recovery may become a reality after a lethal arenavirus infection.

<u>In-situ contracting heart</u> This work was done in collaboration with Col. Barbara Lowry, Pathology Division. The in situ beating hearts from control and Pichinde virus-infected strain 13 guinea pigs, with ventilation maintained by a respirator, were observed and recorded continuously with a video camera. Studies were carried out between 7 and 19 days post-inoculation. The following cardiac abnormalities developed and were divided into four categories (Table 6):

1. Engorgement of superior and inferior vena cava and dilation of the right side of the heart.

2. Weakening of cardiac muscle contractility and slowing of the heart rate.

3. Presence of tiny, epicardial, whitish "markings" 1 to 2 mm, parallel to the left anterior descending coronary artery.

4. The presence of a predominate bulge, filling with a small amount of darkened blood at the apex of the right ventricle synchronized with the heart beat.

It appeared that the right side of the heart suffered more than the left, as indicated by right ventricular dilatation, and pooling of blood in the superior and inferior vena cavae. The formation of a bulge at the apex of the right ventricle further suggested a reduced ejection fraction of blood pumping into the pulmonary artery during systole.

All in situ observations of the beating heart provided additional evidence that the guinea pig's heart was indeed depressed as a result of Pichinde viral infection. These findings are consistent with the in vivo and in vitro studies that cardiac output, work, power, and contractility decreased in strain 13 guinea pigs inoculated with Pichinde virus. The most interesting finding was that the impaired cardiac function after viral infection was not directly related to histopathological changes of the cardiac muscle, but was induced by biochemical alterations within the cardiac muscle, and two concurrently disturbed hemodynamics of the systemic circulation and coronary circulation of the heart.

PRESENTATIONS:

1. **Jahrling, P. B., R. H. Kenyon, and C. J. Peters.** Humoral and cellular immune responses in arenavirus hemorrhagic fever models. Joint Meeting of Royal and American Societies of Tropical Medicine and Hygiene, Baltimore, ML. December 1984.

2. **Jahrling, P. B.** Variability of Lassa virus strains and the relevance to vaccine development. USAMRIID Arenavirus Conference. May 1985.

3. **Kenyon, R. H.** Heterogeneity in the biological properties of Junin virus. USAMRIID Arenavirus Conference. May 1985.

4. **Kenyon, R. H.** Heterogeneity of Junin virus strains. Joint Meeting of the Royal and American Societies of Tropical Medicine & Hygiene, Baltimore, MD. December 1984.

5. **Kenyon, R. H.** Actions of complement on Junin virus and on Junin virus-infected cells. American Society of Virology, Albuquerque, NM. July 1985.

PUBLICATIONS:

1. **Jahrling, P. B., J. D. Frame, S. B. Smith, aud M. I. Monson.** 1985. Endemic Lassa fever in Liberia III. Characterization of Lassa virus isolates. *Trans. R. Soc. Trop. Med. Hyg.* 79:374-379

2. **Jahrling, P. B., and C. J. Peters.** 1985. Arenaviruses, pp. 171-189. *In* E. H. Lennette, (ed.), Laboratory diagnosis of viral infections, Chapter 11. Marcel Dekker, Inc., NY.

3. **Jahrling, P. B., B. S. Niklasson, and J. B. McCormick.** 1985. Early diagnosis of human Lassa fever by ELISA detection of antigen and antibodty. *Lancet* 1:250-252.

4. **Kenyon, R. H., D. E. Green, and C. J. Peters.** 1985. The effect of immunosuppression on experimental Argentine hemorrhagic fever in guinea pigs. *J. Virol.* 53:75-80.

5. **Liu, C. T.** Techniques for isolation and performance of the perfused total working heart. *Am. J. Vet. Res.* 47:(In Press).

LITERATURE CITED

1. **Peters, C. J.** 1984. Arenaviruses. *In* R. B. Belshe (ed.), Textbook of Human Virology. PSG Publishing Company, Inc. Littleton, CO.

2. **Jahrling, P. B., J. D. Frame, S. B. Smith, and M. H. Monson.** 1985. Endemic Lassa fever in Liberia. III. Characterization of Lassa virus isolates. *Trans. R. Soc. Trop. Med. Hyg.* 79:374-379.

3. **Niklasson, B. S., P. B. Jahrling, and C. J. Peters.** 1984. Detection of Lassa virus antigens and Lassa virus-specific immunoglobulins G and M by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 20:239-244.

Table 1. Comparison of human Lassa fever (LF) severity[a] and virulence [b] of Liberian Lassa virus (LV) isolates for strain 2 or 13 guinea pigs

Human LF Patients	Disease Severity	Numbers of LV Isolates: Lethal[a] Strains 2 & 13	Lethal[a] Strain 2 only	Benign[a] Strains 2 & 13
Adult	Death	2	1	1
	Severe	0	6	1
	Mild	0	5	9
Pregnant	Death/Severe	0	2	2
	Mild	-	1	1
Infant	Death/Severe	-	2	2

[a] Severe: > 10 days in hospital, fever > 103°F, shock, and/or hemorrhagic signs. Mild: < 10 days in hospital, fever < 103°F, and no signs of shock, hemorrhage, or other complications.
[b] Isolates classified as lethal if 4 or 5 out of 5 guinea pigs died after 4.1 to 4.8 $\log_{10}$PFU LV inoculation s.c.

Strain	PFU/LD_{50}	Time to death (Mean days) # Dead[a]/10	Guinea Pig Disease[b]	Human Disease Pattern	Pattern
Espindola	5.0×10^{-2}	10	17.3 ± 2.7 hemorrhagic (fatal)	hemorrhagic	severe
Ledesma	3.0×10^{-1}	10	19.0 ± 2.3 hemorrhagic (fatal)	hemorrhagic/neurologic	severe
Romero	2.0×10^{-1}	10	14.5 ± 1.7 (not fatal)	hemorrhagic	mixed
3551	1.6×10^{2}	8	21.1 ± 2.5 hemorrhagic (fatal)	neurologic/hemorrhagic	severe
Coronel	$>10^{5}$	30	neurologic mixed (fatal)	severe	
Suarez	1.0×10^{4}	6	27.8 ± 3.9 (not fatal)	neurologic	mild
XJ-44	$>10^{7}$	-	none	unknown	
Candidate 1	$>10^{7}$	-	none	none	

[a]Guinea pigs inoculated i.p. with 100 LD_{50}, except for those strains where an LD_{50} was not calculated, we inoculated 10^5 PFU.
[b]± S.D.

Table 3. In vitro characteristics of virulent and attenuated strains of Junin virus

	Junin Virus Strain	
In Vitro Test	Virulent	Attenuated
Complement Fixation	No effect	Inactivation
Neutralization	Needs Complement. Most efficient with anti-serum generated against virulent strain.	Complement not needed. Antiserum may be generated against virulent or attenuated strcin.
Infected Cell Lysis	Lysed by complement and antibody generated against virulent virus but not attenuated virus.	Lysed by complement and antibody generated against virulent or attenuated virus.
Fluorescent Antibody Cell Sorting (infected cells)	Cell-surface viral antigen detected by antisera to virulent strains only.	Cell surface viral antigen detected by antisera to virulent or attenuated strains.

Table 4. Effect of ribavirin on guinea pigs infected intraperitoneally with virulent (Romero strain) Junin virus

TreatmentDead/ (Days)	Mean Day- Total	Mean Day Paralysis To-Death	Onset of Total	Paralysis
None	20/20	15.7 $\pm$ 2.0	0/20	-
Ribavirin (-1->14)	8/8	24.9 $\pm$ 2.2	1/8	26
Ribavirin (+7->24)	7/8	23.6 $\pm$ 3.2	3/8	21.3 $\pm$ 0.6
Ribavirin (-1->24)	8/8	25.0 $\pm$ 2.8	4/8	23.5 $\pm$ 1.7
Ribavirin (-1->24)	0/8	-	-	-

Table 5. Bioassay (ileum contractility) and plasma immunoreactivity for sulfidopeptide leukotrienes in control and Pichinde virus-infected guinea pigs.

Techniques	Bioassay response or leukotriene concentration	Uninfected control (n=3)	Days after Pichinde Virus Inoculation 7 (n=5)	11 (n=5)	14 (n=7)
Bioassay[a]	Ileum contractility	+	+	++++[b]	0[c]
	LTE_4 equivalent (ng)	3	4	18	0[d]
Radioimmuno-assay[e]	LTC_4 equivalents (ng/2.5 ml)	<1	<1	1.5	<1[f]

[a]Plasma used = 5 ml (results are expressed as LTE_4 equivalents in smooth muscle contraction).
[b]Plasma used = 2.5 ml.
[c]One sample showed + response.
[d]One sample showed 2 to 5 ng.
[e]Since individual species of sulfidopeptide leukotrienes were not separated, the immunoreactivity is expressed in units of LTC_4 equivalents.
[f]One sample showed 2.4 ng/2.5 ml plasma.

Table 6. Frequency of occurrence of apparently gross lesions of the contracting heart after Pichinde virus infection

Gross Observations	Category No.[a]	Control (N=2)	Day Postinoculation 7 (N=2)	11 (N=3)	13 (N=3)	16 (N=2)	19 (N=3)[b]
Congested Heart	1	0	0	3	2	1	2
Weak Contractility	2	0	0	0	3	2	3
Whitish Lesion	3	0	0	0	0	1	1
Apex Bulge	4	0	0	0	1	2	2

[a]Defined in the text.
[b]One died shortly after thoracotomy.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA 302646	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AE	134
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) (U) Exploratory Research for Protection Against Ebola Virus

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	2.0	693
c. TYPE	d. AMOUNT	86	0.0	0*
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Disease Assessment Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Johnson, E D
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7244
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Lewis, R
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Cosgriff, T M

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Vaccines; (U) Viral Diseases; (U) Lab Animals; (U) Monkey; (U) Guinea Pig; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To ascertain the disease spectrum of Ebola virus, the number of virus strains, and virus reservoirs. To understand the pathogenesis of the disease, permitting rational therapy and immunoprophylaxis for U.S. military personnel stationed in Ebola-endemic areas.

24. (U) Longitudinal epidemiological studies correlate clinical manifestations with seroconversions and identify sites for in-depth ecological studies to recover virus strains from reservoirs.

25. (U) 8410-8509-Seroepidemiological studies indicate that the filovirus group may consist of several serologically cross-reactive, Ebola-like viruses and Marburg virus. Also, human filovirus infections are more common than first expected in endemic areas. The incidence of Ebola infections seems to correlate with the occurrence of the rainy season and be associated with select host-related factors. Lab studies suggest pathogenesis for two prototype filoviruses are different, even though a microangiopathy and myocardial dysfunction occure in both diseases. These differences suggest a requirement for alternative therapeutic measures to control filovirus infections.

"*This research will be part of 871AB in FY 86".

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Wa[illegible]

WORK UNIT NO. 871-AE-134: Exploratory Research for Protection Against Ebola Virus

PRINCIPAL INVESTIGATOR: Eugene D. Johnson, Ph.D.

ASSOCIATE INVESTIGATORS: Richard Lewis, Ph.D.
Thomas M. Cosgriff, COL, M.C.

Background:

The filoviruses - Ebola virus (EBO) and Marburg virus (MBG - form a newly emerging group of negative-stranded RNA viruses endemic to cent[illegible], eastern, and southern Africa. These viruses cause sporadic, isolated cases [illegible] widespread epidemics of severe and often fatal hemorrhagic fever. Mortality and morbidity are high; case mortality has frequently reached levels of 20 to 90% and survivors usually undergo a prolonged convalescence. In many instances, nosocomial infections have been a common consequence of infections in which these agents have not been considered in the differential diagnosis.

The emphasis of the filovirus research project has been: (1) to evaluate the natural threat and impact of this group of viruses in Africa by accurately establishing the incidence and distribution of apparent and inapparent infections and determining epidemiological, ecological, and agent-related factors influencing endemic virus activity; and (2) to alter the highly pathogenic nature of these agents by defining the pathogenic mechanism(s) involved in the disease process and developing therapeutic measures to intervene in the hemorrhagic fever.

Summary:

Cross-sectional seroepidemiological and hospital-based, disease-surveillance studies in endemic areas have indicated that the filoviruses are more active than first expected. Based upon serological results, the incidence of infection in hospitalized fever patients ranges from 10 to 27% during peak periods. Virus activity appears to be influenced by environmental factors: the highest antibody prevalence occurs in the more arid ecological zones, and the incidence of infection is associated with rainy seasons. Host-related factors, such as age and sex, also influence virus activity, but the relative importance of individual factors vary with the culture backgrounds of the affected populations. The importance of agent-related factors is less clear. The high incidence of infection without severe hemorrhagic disease suggests that serologically related, less pathogenic members of the filovirus group may also be active in Africa. These yet unisolated agents may be responsible for cross-immunity protecting against the more pathogenic filoviruses. Laboratory studies with non-human primates showed clearly that the prototype strains of Ebola and Marburg virus can cause severe disease closely resembling that observed during human hemorrhagic fever outbreaks. These findings indicate that the underlying pathogenic mechanism in filovirus infections stems from a multi-system dysfunction resulting from cardiac and hepatic dysfunction and lymphoid tissue necrosis. However, the different pathophysiological features seen in the two diseases reflect the relative effect these agents have on the endothelial and reticuloendothelial cell systems, central in hemostasis. The results suggest

that consideration should be given to developing distinct approaches for therapeutic intervention in filovirus infections.

Progress:

Filovirus field studies Two conditions of endemic filovirus activity were identified: one in which the filovirus antibody prevalence rate is low in the general population, even though nonfatal febrile filovirus-like infections are frequently observed; and one in which the antibody prevalence rate is high and clinical illnesses are documented rarely. Field studies were undertaken to determine factors influencing both conditions.

East Africa A surveillance system for acute fever cases was established in five hospitals in western Kenya, the site of the most recently documented Ebola virus and Marburg virus cases. The system was developed to identify viral hemorrhagic fever cases and define the circumstances surrounding each infection. Day of admission (acute phase) and day of discharge (convalescent phase) serum samples were collected from each patient with a viral-like illness. Virus isolation attempts were made in standard cell culture systems on acute-phase samples, and comparative antibody titrations were conducted on paired acute- and convalescent-phase sera. Limited follow-up studies were undertaken in the villages of the most likely viral hemorrhagic fever cases, those cases that seroconverted or experienced significant rises in antibody titer during the clinical stages of their illness.

A total of 471 cases were detected within the first 18 months of the study. The most common symptoms were high fever, headache, and sore throat; chest, abdominal, joint, or back pain; as well as diarrhea and/or vomiting, with or without blood. Of these human cases, 53.8% were male and 46.2% were female. No significant differences were observed in the sex-adjusted age distribution for either sex. The mean age was 21.4 years (range: 3 months to 70 years).

When screened in an indirect immunofluorescent antibody assay, 10.4% (49/471) of the collected sera reacted with Ebola virus antigens. Six tenths (0.6) per cent (3/471) reacted with Marburg virus antigens. This high Ebola virus reactivity in fever patients was unexpected since the overall prevalence rate in the non-hospitalized population is less than 2%.

Paired sera were collected from 24 of the 49 seropositives: 13 (54.2%) patients seroconverted, and 11 (45.8%) exhibited rising antibody titers during their illness. Five of the 11 fever cases who had been ill for at least five days before hospitalization were discharged with significant antibody titers of 1/128 or 1/256. The mortality rates in the antibody-positive and -negative fever patients were surprisingly low: 4.1% (2/49) and 6.8% (29/423), respectively.

Follow-up studies were conducted on five of the 11 patients who demonstrated significant rises in Ebola virus-reactive antibody titers. Two patients, a six-year-old female and a 16-year-old male, vomited blood or had bloody diarrhea during the clinical stages of their infection. The follow-up studies did not yield evidence of additional cases or a significant number of antibody-positive family members or neighbors. Only two additional antibody-positive individuals were identified. The sex similarities between the two antibody-positives and the male index case suggest an association, but further studies are required. Surprisingly, three of the five index cases had experienced significant drops in antibody titer (> fourfold) when reevaluated four or six months post-hospitalization.

Though follow-up studies did not turn up risk factors for filovirus infection, analysis of the antibody prevalence indicated that environment- and host-related factors influenced virus activity. An increase in antibody-positive fever cases occurred at the end of each rainy season. In east Africa, the long rains occur between March and May, while the short rains occur in October and November. During this five-month period, none of the fever patients (0/137) was found seropositive. However, approximately 9.2% (13/140) of the patients in June, July, and August; and 26.7% (3/30) of the fever cases in December and January experienced filovirus-like infections. When the prevalences rates in fever cases were adjusted for sex and age, both characteristics were found to be important risk factors. Significant antibody levels were observed in the younger age groups (6.8%, 8/117), but the highest rates (16.7%, 14/84) were observed in the 20- to 30-year-old age groups. In this group, the rate in males (22.0%, 11/50) was approximately threefold higher than the rate in the corresponding female population (8.8%, 3/34).

Collectively, the findings indicate that the filoviruses are capable of causing significant illness in select populations in western Kenya. Though the agent responsible for the febrile illness has not been identified, the data provided clear insight into the epidemiology and ecology of this virus. In light of the highly pathogenic nature of the known members of the filovirus group, isolation of this seemingly less pathogenic agent from clinical specimens seems warranted.

Central Africa Cross-sectional seroepidemiological surveys were conducted in the arid scrub area of the Vakaga district, Central African Republic (CAR). These serosurveys were undertaken to confirm and extend previous observations that indicated endemic filovirus activity in central Africa differed significantly from similar activity in east Africa. Preliminary serosurveys have shown that virus activity, as measured by filovirus-reactive antibody prevalence, was higher along the CAR's frontier with Chad and Sudan than in any other African region, and was confined to select female populations and was distributed focally among select villages.

Samples (515) were collected from four villages located in either central or eastern Vakaga; 17.0% (88/515) were found to be seropositive, confirming previous findings (14.2%, 105/740). Again there was a strong tendency toward a higher antibody prevalence rate among Moslem females (20.5%, 40/195) than among Moslem males (12.5%, 30/241). Interestingly, this trend seemed to be reversed in the only Vakaga Christian village; males showed a somewhat higher prevalence rate (29.0%, 11/38) than females (17.0%, 7/41). Additional samples are required to confirm this difference between Christian and Moslem villages.

Ebola virus seemed to be the most active filovirus (Ebola: 15.0%, 77/515; Marburg: 3.1%, 16/515). Activity was similar in villages from central (15%, 41/287) and eastern (21%, 47/228) regions; this suggests that Ebola virus was uniformly active in these two areas but active in only select villages in western Vakaga (Sikikide: 17.2%, 17/99; Armadjedi: 3.8%, 3/80).

The level of Marburg virus activity was significantly higher in the central Vakaga (3.4%, 15/320) than in either eastern Vakaga (0.4%, 1/228) or any other region of Africa. Marburg virus antibody positives (31.3% [5/16]) were also seroreactive with the non-cross-reacting Ebola virus, suggesting shared risk factors for these two virus infections in the western Vakaga.

The serological surveys have defined a unique area in central Africa where filoviruses are active. An association between increased seroconversions and the rainy seasons has not been generated. It is interesting that virus activity in the arid Vakaga is clustered in the western region along the Bohr Oulou river. This suggests that environmental factors also influence virus distribution in the CAR. Possibly, social and behavioral factors shared by a select population influence preferential exposure to vector and host species present in the more moist river basin. Further studies will be required to determine the actual mechanism by which filoviruses are maintained and transmitted in central Africa without causing demonstrable clinical disease.

Filovirus pathogenesis Epidemiological investigations suggest that unidentified filovirus(s) are active in endemic areas. The lack of significant hemorrhagic fever suggests that, presumably, serologically cross-reacting viruses may limit the expression of the more highly pathogenic filovirus strains.

Clearly, laboratory studies indicate that the prototype Ebola virus and Marburg virus strains are capable of causing severe, uniformly fatal disease in non-human primates. The disease closely resembles that seen in humans.

As expected, animals inoculated with low levels of virus, 10^1 to 10^2 PFU, developed a hemorrhagic diathesis within six to eight days. Both prothrombin and partial thromboplastin times were prolonged significantly, indicating that all coagulation pathways were altered. Ebola virus infections affected the levels of intrinsic pathway factors 12 and 9, and common pathway factors 8, 10, and 2. Extrinsic pathway factors for common pathway 5 were not affected. Marburg virus infections induced a different pattern. Extrinsic pathway factor 7 and common pathway factors 5, 8, and 2 were suppressed without having any effect on the levels of intrinsic pathway factors 12 or 11.

Suppressed coagulation factor activity occurred rapidly during both infections. Coagulation factor 8 was suppressed to 20% of activity levels by the onset of clinical disease or fever. In Ebola virus infections, the fall in factor 8 was paralleled by a fall in intrinsic factor 12 and platelets, suggesting that the intrinsic coagulation pathway was stimulated early in Ebola hemorrhagic fever. Extrinsic factor 7 levels remained unchanged through the infection. The rapid fall in factor 8 may reflect early endothelial cell dysfunction rather than decreased factor synthesis in the liver, since common pathway factor 5 was not suppressed. The importance of endothelial cell dysfunction or damage to the decline of factor 8 levels in Marburg virus infections was less clear. The simultaneous decline in factors 7 and 8 after the appearance of circulating Marburg virus may reflect the activation of the extrinsic coagulation pathway. This may be due to reticuloendothelial cell involvement rather than endothelial cell dysfunction.

As in human disease, elevated levels of fibrin degradation products (FDP) were present in the blood of Marburg virus-inoculated animals. The rise in FDP levels coincided with a marked decrease in fibrinogen levels. Though a hypofibrinogenemia also occurred in Ebola virus infections, it was not accompanied by circulating FDP. The severe hypofibrinogenemia pan-suppression of coagulative factors, and suppression of anti-thrombin III, as well as the presence of an activated fibrinolytic system, suggest that an overwhelming consumptive coagulopathy plays an important part in the pathogenesis of Marburg virus disease. However, in other models, a thrombocytopenia is a stretching feature. The lack of a thrombocytopenia in hemorrhagic Marburg virus infections may reflect virus-associated platelet dysfunction.

As a histological correlate, lesions were found consistently in the heart, liver, and kidneys of primates. All infections resulted in consistent heart lesions. Myofiber swelling and uniformly distributed vacuolar changes in the cytoplasm, or cardiac majority were common. Numerous distinct myocardial infarcts, and pericardial and multifocal myocardial hemorrhage were common features of Marburg virus infections. This was the first direct evidence that the heart is affected acutely. Similar histopathological lesions have not been described in humans, though there are several reports of filovirus-associated myocarditis during Ebola virus and Marburg virus epidemics.

Histopathological evidence of the hemorrhagic diathesis was present in many tissues from Marburg-infected animals. Microscopic, hemorrhagic, fibrin thrombi were present in the skin, skeletal muscles, brains, and urinary bladders. The lack of necrothrombi was surprising in light of the presence of an active fibrinolytic system, as indicated by elevated levels of FDP.

Although consistent with human disease, the presence of moderate histopathological lesions in the liver, does not support liver failure as the sole contributing factor to the hemorrhagic diathesis in Ebola hemorrhagic fever or Marburg virus disease. Typically, the liver lesions consisted of a fatty degenerative, widely distributed, but focal, necrosis of the parenchyma. This necrosis was accompanied by an inflammatory reaction. The most striking reaction was a severe leukocyte margination in Ebola virus-infected livers.

The kidney lesions were also unimpressive. Moderate fibrin deposits were observed consistently in Ebola virus-infected kidneys. These deposits could also be consistent with a hemorrhagic diathesis. But the occurrence of a hypofibrinogenemia with fibrin deposits in the tissues, without elevated circulating levels of FDP, may reflect an Ebola virus-associated alternative in the fibrinolytic system. The only histopathological evidence of a hemorrhagic diathesis in Ebola virus-infected animals was the ring hemorrhage observed surrounding necrotic germinal centers in the spleen. The hemorrhage was accompanied by cell depletion and accumulation of proteinaceous material in red pulp areas. Similar necrotic germinal centers, depleted of lymphocytes, were also present in Marburg virus disease. The severity and nature of the lymphoid tissue necrosis suggests that filovirus infection may affect directly the reticuloendothelial cell system. Such an effect could dramatically alter immune coagulation and fibrinolytic systems.

PRESENTATIONS: None

PUBLICATIONS: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 302650	85 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	H. TERM	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M152770A871	AF	135
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)

(U) Exploratory Research for Protection Against Chikungunya

12. SUBJECT AREAS

0605 Clinical Medicine; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	8409	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (in thousands)
85	0.0	0
86	0.0	0*

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Disease	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Lupton, H W
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2405
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Levitt, N H
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Ramsburg, H H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Vaccines; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop and produce inactivated and attenuated Chikungunya vaccines which may then be combined or administered sequentially to at-risk militatry forces for prophylaxis after geographical or BW exposure.

24. (U) Chikungunya virus is propagated in certified diploid cell cultures and inactivated with formalin or selected for attenuation. Products will be safety tested to assure freedom from contamination with adventitious agents. Efficacy is determined by subsequent challenge, or by determination of serological conversion.

25. (U) Using CHIK 181/Clone 25, a vaccine master seed was prepared at USAMRIID and transferred to The Salk Institute (TSI) where CHIK vaccine production seed and three lots of live, attenuated candidate vaccine were produced. Safety tests required by the Food and Drug Administration have been conducted with final tests in progress at the time of writing. Oligonucleotide mapping demonstrated a biochemical difference involving the largest oligonucleotide, which was present in the CHIK parent, but absent in 181/Clone 25, thus, enhancing the evidence that genetic change(s) occurred during the cloning/selection procedures used to develop the candidate vaccine. In addition, in vitro cross neutralization studies were conducted employing antiserum prepared against a prototype CHIK vaccine and O'nyong nyong, Mayaro, and Ross River viruses. The CHIK antiserum was found to neutralize O'nyong nyong to a greater degree than Mayaro, with no cross-neutralization observed with Ross River virus. An IND application is in preparation.

*This research has been switched to 6.2 funding and will be part of Work Unit 807AC in FY86.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AF-135: Exploratory Research For Protection Against Chikungunya

PRINCIPAL INVESTIGATOR: H. W. Lupton, COL, Ph.D.

ASSOCIATE INVESTIGATORS: N. H. Levitt, Ph.D.
H. H. Ramsburg, M.A.

Background:

Chikungunya (CHIK), an arthropod-borne virus producing a dengue-like illness in man, is disseminated widely throughout Africa, southeast Asia, the Western Pacific, and India. Despite their widespread geographic distribution, individual strains of the CHIK virus are closely related antigenically (1,2), thereby allowing a vaccine prepared with one CHIK strain to provide broad-spectrum protection against heterotypic strains of this virus. Moreover, a vaccine has further potential of protecting vaccinees against related viruses such as O'nyong nyong, Mayaro, and Ross River. Numerous reports of epidemics (3,4,5,6,7,8) as well as infection among laboratory personnel working with CHIK virus (9,10,11) have indicated the need for a safe and efficacious vaccine.

By using CHIK strain 15661, a 1962 southeast Asian human isolate, the Walter Reed Army Institute of Research (WRAIR), developed a formalin-inactivated, human-use vaccine, made in Green Monkey kidney cell culture, that was tested clinically in volunteers and at-risk personnel (12). Because of the extensive research into its biological and immunological characteristics, CHIK strain 15561 was chosen as the seed virus for developing a live, attenuated vaccine for human use. A candidate vaccine virus strain was developed by subjecting CHIK strain 15561 to a series of plaque-to-plaque passages in vaccine quality MRC-5 cells. A master seed, production seed, and three lots of vaccine have been produced, with safety testing now in progress.

Summary:

By using CHIK 181/Clone 25, a vaccine master seed was prepared at USAMRIID and transferred to The Salk Institute (TSI), Swiftwater, PA, where CHIK vaccine production seed and three lots of live, attenuated candidate vaccine were produced. Safety tests required by the Food and Drug Administration have been conducted with final tests in progress at the time of writing. Oligonucleotide mapping demonstrated a biochemical difference involving the largest oligonucleotide, which was present in the CHIK parent, but absent in 181/Clone 25, thus, enhancing the evidence that genetic change(s) occurred during the cloning/selection procedures used to develop the candidate vaccine. In addition, in vitro cross-neutralization studies were conducted employing antiserum prepared against a prototype CHIK vaccine and O'nyong nyong, Mayaro, and Ross River viruses. The CHIK antiserum was found to neutralize O'nyong nyong to a greater degree than Mayaro, with no cross-neutralization observed with Ross River virus. An IND application is in preparation.

Progress:

During this reporting period, master seed, production seed, and three lots of vaccine were produced with our vaccine candidate, CHIK 181/Clone 25 virus. Master seed was produced at USAMRIID by inoculating four T-150 flasks of vaccine-quality, MRC-5 cell cultures with 3 $\log_{10}$ PFU of virus. At 42 h post-inoculation, 200 ml of master seed was harvested and found to have a titer of 8.9 $\log_{10}$ PFU/ml. Results of sterility tests demonstrated the absence of bacterial contamination. The master seed was transported to The Salk Institute where production seed and vaccine were prepared under the same growth conditions as that described for master seed. One liter of production seed with a titer of 9.3 $\log_{10}$ PFU/ml was harvested. Three one-liter lots of vaccine were subsequently prepared with titers of 9.1, 9.2, and 9.3 $\log_{10}$ PFU/ml, respectively.

Prior to lyophilization, lot 1 vaccine concentrate was diluted to contain 5.0 $\log_{10}$ PFU/0.5 ml, a dose derived from results obtained from a monkey protection study employing varying doses of vaccine (13). This vaccine maintained its titer, plaque size, and homogeneity during the lyophilization process.

Safety testing of production seed and lot 1 vaccine required by FDA has been completed or tests are ongoing. Sterility (bacteria and mycoplasma), animal inoculation (rabbits, adult mice, suckling mice, and guinea pigs), monkey neurovirulence (gross and histopathology), and reverse transcriptase tests are complete, while tests for tissue culture (viral adventitious agents) and monkey neurovirulence (histopathology) are in progress.

Oligonucleotide mapping was applied to the CHIK parent strain 15561 and the derived candidate vaccine virus, CHIK 181/Clone 25, to detect possible genetic markers of attenuation. Viruses were propagated by infecting MRC-5 cell monolayers at an input multiplicity of 0.02 PFU per cell. Following incubation at 35°C for 48 h in the presence of 150 Ci/ml [^{32}P] orthophosphate, viruses were harvested, concentrated, and purified as previously described (14). The intrinsically labeled genomic RNAs were then extracted from gradient purified virus, digested with RNase T_1 and analyzed by two-dimensional polyacrylamide gel electrophoresis (14).

The oligonucleotide fingerprint patterns obtained clearly distinguished genetic differences between parent and candidate vaccine strain (Figure 1). The most striking difference involved the largest oligonucleotide (approximately 40 nucleotides long), which was present in the CHIK parent strain but absent in the prototype candidate vaccine virus. The other noticeable difference involved the vertical shift of a smaller uridine-rich oligonucleotide. These reproducible alterations enhance the evidence that a major genetic change occurred during the extensive cloning/selection process.

Since antigenic similarities exist between certain alphaviruses such as CHIK, O'nyong nyong, Mayaro, and Ross River, we decided to determine if our CHIK vaccine would ultimately cross-protect vaccinees against these related viruses. Initial in vitro cross-neutralization studies were performed using monkey antiserum prepared against a prototype vaccine and O'nyong nyong, Mayaro, and Ross River viruses. Plaque-reduction neutralization antibody titers ($PRNT_{80}$) against O'nyong nyong and Mayaro viruses were 1:80 and 1:10, respectively, while the titer against Ross River was <1:10. These results are encouraging in that cross-neutralization occurred with both O'nyong nyong and Mayaro viruses and indicates that cross-protection studies in animals are warranted.

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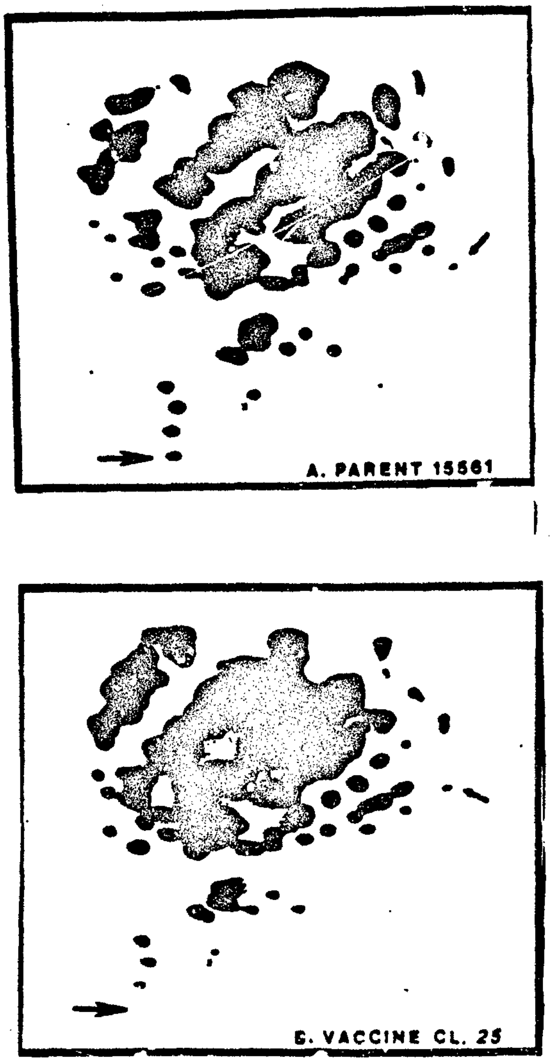

Figure 1. Oligonucleotide fingerprints of (A) CHIK parent 15561 and (b) derived candidate vaccine virus CL 25. The ribonuclease T_1-resistant oligonucleotides were derived from ^{32}P-labeled virion RNA and separated by two-dimensional gel electrophoresis. Migration in the first dimension is from left to right, and in the second dimension, from bottom to top. The positions of the two dye markers, bromophenol blue (upper) and xylene cyanol FF (lower) are indicated (X). Fingerprint (B) is missing the largest oligonucleotide which is present in (A), indicated by the large arrows. The small arrows point out the smaller oligonucleotide which exhibits a vertical shift of position.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 302648	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY H. TERM	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AG	136
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Exploratory Research for Protection Against Rift Valley Fever Virus

12. SUBJECT AREAS
0605 Clinical Medicine; 1503 Defense; 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	0.3	4.4
c. TYPE	d. AMOUNT	86	0.0	4.4
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Dalrymple, J M
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2775
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available)
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine ;(U) Lab Animals; (U) Vaccines; U) Mice;(U) Rift Valley Fever Virus; (U) Therapy; (U) Prophylaxis; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To explore the basic technology to produce novel vaccines for protection of U.S. military personnel in exotic locations. Rift Valley fever virus (RVFV) will be used as a prototype for these studies.

24. (U) The relevant portion of the genome is determined to be followed by construction of a DNA copy. Cloning and sequencing attempts are made to induce synthesis of immunogenic products in various expression systems.

25. (U) 8410 - 8509 - Partial coding sequences of RVFV glycoprotein genes were incorporated into a bacterial plasmid expression system. Introduction of these plasmids into *E. coli* allowed the regulated, high-level expression of RVFV glycoprotein analogues. Partially purified polypeptides from these systems were used with adjuvants to immunize mice. A high percentage of mice were protected from lethal challenge with RVFV although only marginal titers of neutralizing antibody resulted from the immunization. Recombinant vaccinia viruses containing the genes responsible for both RVFV envelope glycoproteins were also produced. These recombinant vaccinia viruses were evaluated similarly in the mouse-challenge system with various routes for administration of the recombinant vaccine. High-titer neutralizing antibody and high-level protection followed immunization with these vaccines. Preliminary data from the immunization of pregnant sheep suggest that recombinant vaccinia virus vaccines may well be effective in preventing abortion after RVFV infection.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AG-136: (U) Exploratory Research for Protection Against Rift Valley Fever Virus

PRINCIPAL INVESTIGATOR: Joel M. Dalrymple, Ph.D.

Background

Rift Valley fever virus (RVFV) meets many of the requirements for military medical research interest because of the disease threat to troops operating in endemic areas and potential strategic use of RVFV as a BW agent. Although the existing RVFV vaccine has been shown safe and effective in man, the limitations for its widespread use have been detailed in previous reports and dictate the continued investigation of an improved product. This research has been directed specifically toward the application of recent techniques of biotechnology to virus vaccine development. The expected results will include both an improved vaccine for protection against Rift Valley fever and a well-defined, technical strategy for subsequent development of successful vaccines for other viral pathogens with similar characteristics.

The molecular approach to vaccine development was initiated with an in-depth characterization of RVFV and antigenic analysis of virion components. Subsequent isolation, identification, and molecular cloning of the genes encoding major viral antigens was accomplished in collaboration with USAMRDC contractors and is detailed similarly in previous reports. Recent studies have emphasized the expression of these genes in both bacterial and viral expression systems. The efforts described in this report were directed toward estimating the potential of these products as vaccine candidates based upon their ability to induce virus neutralizing antibody and protect against infection and/or disease.

Summary:

Partial coding sequences of RVFV glycoprotein genes were incorporated into a bacterial plasmid expression system. Introduction of these plasmids into *E. coli* allowed the regulated, high-level expression of RVFV glycoprotein analogues. Partially purified polypeptides from these systems were used with adjuvants to immunize mice. A high percentage of mice were protected from lethal challenge with RVFV although only marginal titers of neutralizing antibody resulted from the immunization.

Recombinant vaccinia viruses containing the genes responsible for both RVFV envelope glycoproteins were also produced. These recombinant vaccinia viruses were evaluated similarly in the mouse-challenge system with various routes for administration of the recombinant vaccine. High-titer neutralizing antibody and high-level protection followed immunization with these vaccines. Preliminary data from the immunization of pregnant sheep suggest that recombinant vaccinia virus vaccines may well be effective in preventing abortion after RVFV infection.

Progress:

Expression of RVFV gene segments in bacterial expression systems previously employed the expression of a particular RVFV-coded peptide linked to the bacterial enzyme β-galactosidase. These studies suggested that such fusion proteins were not good immunogens in that they did not induce the production of high-titer neutralizing antibody, even though large quantities of protein were used to immunize. Mouse protection experiments with these same fusion proteins indicated that the G2 protein region of the genome provided better immunogenic potential than the G1 protein coding region.

An objective of these experiments was to express defined regions of both the G2 and G1 protein genes independent of any direct association with β-galactosidase, which was presumed from previous experiments to be deleterious to maximum antibody formation. Although many different constructs and products were examined, only the most successful peptide antigen will be described. The plasmid construct used for expression (ptRV-BH4DT1) contained the *tac* promoter; the ribosome binding site and first 23 amino acids of the phage *cro* gene; the RVFV sequences between nucleotides 302 and 1197, flanked by linker sequences, followed by expression vector translational stop codons; and the T1 transcriptional terminator from the rrnB operon. In response to induction of the promoter, this plasmid directed the synthesis of a *cro*-G2 glycoprotein fusion polypeptide of molecular weight 36,000.

The expressed protein, designated BH4D, represented the amino terminal 49% of the mature G2 glycoprotein sequence as well as 59 amino acids of RVFV polypeptide preceding mature protein sequences. The polypeptide was expressed to high levels and was accumulated as an insoluble aggregate representing 5 to 10% of the total bacterial protein. Preparation of a partially purified product for the immunization of mice involved disruption of the expressing cells with lysozyme, detergents, and sonication, followed by low-speed centrifugation to yield a BH4D-enriched protein pellet. The pellet fraction was solubilized in 9 M urea and 2% 2-mercaptoethanol by heating to 100°C followed by dialysis to remove the denaturant. Various amounts of this preparation were diluted and mixed with the adjuvant, pluronic polyol L121; animals were injected i.m.

Two strains of mice, ICR outbred females and C57 Black 6J strain males at 4 to 6 weeks-of-age, were injected on day 0 and boosted with an injection containing one-half of the original protein concentration on day 21. Ten days after the booster injection, all animals plus unimmunized controls were challenged i.p. with 10 to 50 LD_{50} of RVFV. Data resulting from these challenge experiments are presented in Table 1.

Unimmunized control animals all died with the virus challenge dose; the C57 Black 6J mice dying between 3 to 5 days postinfection with acute liver necrosis, and the outbred ICR mice dying between 5 to 13 days, primarily with signs of encephalitis. The immunizing doses employed represented the maximum possible with the relatively impure protein preparations. Higher doses approaching 1 milligram of protein appeared to contain endotoxin activity that resulted in the death of a significant number of the animals. The plaque-reduction neutralization titers (PRNT) or pre-challenge sera were quite low, even though other studies have clearly demonstrated that epitopes responsible for both neutralization and protection reside in the same region of the native G2 glycoprotein represented by the BH4D. The observation that a significant number of these animals were protected from lethal challenge suggests future experiments with bacterially expressed proteins; however, different expression systems as well as improved extraction and purification procedures will probably be required for success.

An alternative to the expression of RVFV genes in bacteria employed the insertion of RVFV genes into vaccinia virus and the use of the resultant recombinant vaccinia as a live virus vaccine in experimental animals. Various gene constructs were employed in initial experiments; however, only the more successful results that employed a near full-length, M-segment gene construction will be discussed. Cleavage of a bacterial plasmid containing the entire M-segment sequence of RVFV resulted in a 3695 base-pair fragment from nucleotide positions 177 to 3872 containing the entire coding regions for both glycoproteins G2 and G1. This fragment was ligated to polylinkers and inserted into a plasmid (pGS20) adjacent to the vaccinia 7.5-K gene promoter and transcriptional start site. This, in turn, was flanked on both sides by portions of the vaccinia thymidine kinase gene. This plasmid was used to transfect mammalian cell cultures infected with vaccinia virus. Recombinant vaccinia viruses containing the RVFV gene insert in the vaccinia thymidine kinase gene region were selected, plaque-purified and used for subsequent animal infection experiments.

A recombinant vaccinia virus containing the near full-length transcript of RVFV M-segment (VRV-5) inserted into the WR strain of vaccinia virus was superior to other recombinants and therefore used for all experiments reported. Recombinant VRV-5 was demonstrated to be capable of expressing RVFV antigens in infected cells by labeling with ^{35}S-methionine and subsequent immune precipitation of cell lysates with RVFV hyperimmune mouse ascitic fluid. Both envelope glycoproteins were radiolabeled and precipitated with the immune ascitic fluids. These results differed from control cultures infected with RVFV in that no nucleocapsid protein was produced; the gene for nucleocapsid protein resides on another segment of RVFV and was not included in the VRV-5 recombinant.

To investigate the immunogenic potential of the recombinant vaccinia virus VRV-5, ICR and C57 Black 6J mice were immunized and subjected to the virulent RVFV challenge test similar to the experiments described for bacterially expressed proteins. The results of a typical experiment are presented in Table 2.

Mice were immunized by a single administration of vaccinia virus by the routes indicated. All mice were bled at 21 days after vaccination and challenged with 10 to 50 LD_{50} virulent RVFV i.p. The parent WR strain of vaccinia virus without genetic modification induced some mortality in the mice. Highest mortality was associated with the intranasal, and to a lesser extent, the intravenous route. This observation was not entirely unexpected, since the WR strain of vaccinia originated from the laboratory selection for properties of mouse neurovirulence. In other experiments involving intracerebral inoculation, this virus kills mice at levels approaching 1 to 5 PFU. Although the occasional animal inoculated with unmodified vaccinia survived lethal RVFV challenge, there was no significant protection.

No mortality was observed after vaccination with the genetically modified VRV-5 vaccinia virus by any route, suggesting that the insertion of foreign genetic elements into the thymidine kinase locus of WR vaccinia had modified the mouse virulence characteristics of the parent virus. In addition, relatively high levels of neutralizing antibody were detected in the majority of the animals. Virtually all of the immunized animals were protected from virulent RVFV challenge. The three animals that died in the intranasal group had lower antibody titers.

Results with recombinant vaccinia virus containing RVFV envelope glycoprotein genes suggest that an improved vaccine for Rift Valley fever may be possible by using modifications of this procedure. In preliminary experiments still in progress, the VRV-5 recombinant vaccinia virus has been used to immunize pregnant ewes. Pregnant sheep abort at a high frequency after infection with RVFV and therefore represent a severe challenge of the efficacy of the proposed recombinant vaccine. All 11 control sheep immunized with unmodified WR vaccinia virus aborted within 8 days after challenge with virulent RVFV; however, 7 of 11 ewes immunized with the recombinant VRV-5 were protected and gave birth to living, healthy lambs. A full description of these experiments must await additional data analysis and evaluation.

The utilization of bacterially expressed RVFV proteins as immunogens and vaccine candidates obviously requires further investigation but shows early promise in that some mouse protection was observed. The advantages of the bacterial systems are numerous in that the techniques for manipulating genetic elements in bacteria are well described, and many of the factors governing expression and control are known. The recombinant vaccinia viruses described offer considerable promise for future vaccine development; however, they too require additional research since the characteristics of genetically modified vaccinia viruses have not yet been completely described, and the various factors governing antigen expression and induction of the immune response require further definition.

PRESENTATIONS

1. **Collette, M. S., K. Keegan, S-L. Hu, P. Sridhar, A. F. Purchio, W. H. Ennis, and J. M. Dalrymple.** Protective subunit immunogens of Rift Valley fever virus from bacteria and recombinant vaccinia virus. International meeting on Negative Strand Viruses, Cambridge, England, September 1985.

PUBLICATIONS

1. **Collette, M. S., A. F. Purchio, K. Keegan, S. Frazier, W. Hays, D. Anderson, M. Parker, C. Schmaljohn, J. Schmidt, and J. M. Dalrymple** 1985. Complete nucleotide sequence of the M segment of Rift Valley fever virus. *Virology* 144:228-245.

2. **Ihara, T., J. F. Smith, J. M. Dalrymple, and D. H. L. Bishop.** 1985. Complete sequences of the glycoproteins and M RNA Punta Toro phlebovirus compared to those of Rift Valley fever virus. *Virology* 144:246-259.

Table 1. Challenge of immunized mice and unimmunized control animals with 10 to LD_{50} Rift Valley fever virus

Dose (μg)	PRNT	Protection Survivors/Total	% Protection
100	(10-40)	14/20	70
200	(10-40)	9/15	60
500	(10)	5/ 9	56

Table 2 Immunization of mice with RVFV M segment recombinant vaccinia virus

Immunizing Virus	Route	Vaccinia Mortality	PRNT	Protection (Survivors/Total	% Protection
WR strain	Footpad	0/7	-	1/7	14
	Intranasal	7/7	-	-	-
	Intraveneous	3/7	-	1/4	25
	Scarified tail	1/7	-	0/6	0
VRV-5	Footpad	0/30	(160-2560)	30/30	100
	Intranasal	0/30	(40-2560)	27/30	90
	Intraveneous	0/30	(40- 640)	30/30	100
	Scarified tail	0/30	(160-2560)	30/30	100

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 3814	85 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	H. TERM	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AJ	138
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Aerosol Studies on Agents of Biological Origin

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	85 10	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	2.9	838
86	0.0	0

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Airborne Diseases Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Stephenson, E H
d. TELEPHONE NUMBER (include area code) 301-663-7453	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Anderson, A O
MILITARY/CIVILIAN APPLICATION M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Berendt, R F

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Military Medicine;(U)BW Defense;(U)Bacteria;(U)Lab Animals;(U)Guinea Pigs;(U)Hamsters;(U)Virus;(U)Aerosols;(U)Mice;(U)RAD I

23. TECHNICAL OBJECTIVE 24 APPROACH 25 PROGRESS (Precede text of each with Security Classification Code)

23. (U) Define the respiratory infectivity or toxicity potential of agents of potential BW importance; elucidate pathogenesis of infections or intoxications induced with aerosols, to include determination of the sequence of events leading to protective immunity. Data obtained will provide the bases for evaluation of prophylactic and therapeutic regimens developed to protect deployed US forces.

24. (U) Develop animal models and define the clinical, pathological, and immunological changes during infections. Characterize immune defenses within the respiratory tract. Information is used to provide bases to determine the efficacy of vaccination and therapy procedures.

25. (U) 8410-8509 - In the BALB/cJ-Rift Valley fever (RVF) virus model, mucosal priming with inactivated virus vaccine and an immunomodulator and with virus vaccine alone reduced or prevented aerosol-acquired encephalitis, yet did not prevent hepatitis. Priming by s.c. injection with vaccine plus immunomodulator yielded mucosal and systemic protective immunity. An in vitro system using biotinylated virus was developed to evaluate viral tropisms. Diacetoxyscirpenol (DAS) was toxic for mice at 50 mg/ml in aerosol when dissolved in ethanol (ETOH), but not when dissolved in DMSO. Lethality occurred in F-344 rats to aerosols of DAS in either ETOH or DMSO (50 mg/ml). Pathologic lesions included lymphoid necrosis and cellular degeneration, plus necrosis of zona fasciculata of the adrenals.

Continued under DA302630, DA302646 and DAOG3810.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AJ-138: Aerosol Studies on Agents of Biological Origin

PRINCIPAL INVESTIGATOR: E. H. Stephenson, D.V.M., Ph.D., COL, VC

ASSOCIATE INVESTIGATORS: A. O. Anderson, M.D., LTC, MC
R. F. Berendt, Ph.D.
A. D. King, D.V.M., CPT, VC

Background:

Any microorganism to be employed as a BW agent against US forces must be infective or toxic via the respiratory tract. Investigations are designed to define the respiratory infectivity or toxicity potential of candidate agents. The pathogenesis of infection and intoxications induced with aerosols is elucidated, to include determination of the sequence of events leading to protective immunity. Particular emphasis is on the role of respiratory mucosal immunity in the inducement of protection against an airborne challenge with toxins or microbes.

Summary:

Major accomplishments made within the work unit were in the following areas:

1) In the BALB/cJ mouse - Rift Valley fever virus (RVFV) model, data suggest that intraduodenal (mucosal) priming with inactivated RVF virus vaccine and Avridine, an immunomodulator, and with RVF virus vaccine alone is effective in reducing or preventing aerosol-acquired encephalitis, even though this route of priming is ineffective in preventing hepatitis. Priming by s.c. injection with vaccine plus Avridine results in mucosal, as well as systemic, protective immunity. Apparently Avridine is responsible for producing a more diverse response that includes mucosal immunity. Additionally, extramural collaboration was continued to study the effects of immunomodulators on cells of the immune system.

2) An in vitro system for evaluating viral tropisms was developed. Using biotinylated Venezuelan encephalomyelitis virus (VEE) virus on thin sections of tissue provided a rapid and simple method to identify virus receptor sites. This will assist in the determination of the site of entry of virus into a target organ. Preliminary data indicate one of the portals of entry for VEE virus into the central nervous system (CNS) is through myelinated axons.

3) Diacetoxyscirpenol (DAS), at a concentration of 50 mg/ml of DMSO, was not toxic for mice. When dissolved in ethanol (ETOH), DAS caused a lethal response. Histologically, lymphoid necrosis and cellular degeneration were observed, as well as necrosis of the zona fasciculata of the adrenals. Lethality was seen in F-344 rats to aerosols of DAS in either DMSO or ETOH (50 mg/ml diluent). Preliminary data suggest that the LD_{50} of ETOH-DAS for F-344 rats approximates 4.0 mg/kg. Pathologic lesions were similar to those seen in mice, although more severe.

Progress:

Mucosal Immunity Studies were continued to compare intraduodenal (i.d.) mucosal priming with parenteral (subcutaneous) priming. Groups of BALB/cJ were primed with formaldehyde-inactivated RVFV vaccine, either with or without the immunomodulator Avridine (Table 1). Each group was subdivided; one subgroup was challenged with s.c. injection of virulent RVFV, and the second subgroup was exposed to airborne challenge. Controls included untreated and immunomodulator-treated mice that were sham-inoculated via the two routes and challenged by s.c. injection or aerosol exposure.

Best survival was attained in the groups that were primed s.c. and challenged by that route. There was 40% survival in the group that was primed s.c. with vaccine plus Avridine and challenged by aerosol. This group had 100% survival through day 6, but, as anticipated, there were late deaths due to encephalitis and interstitial pneumonitis. Fifteen percent of the animals treated only with s.c. Avridine (immunomodulator) survived and none of the other groups exhibited long-term survival to a challenge dose of 150 LD_{50}. Histopathology of autopsied moribund mice clearly showed an association between late encephalitis and s.c. vaccination, and between late hepatitis and markedly reduced encephalitis in the i.d. vaccinated groups (Table 2). However, virtually all late survivors of an aerosol challenge among the untreated controls exhibited encephalitis and histopathology, indicating hepatitis.

These data suggest that the mucosal priming with RVFV vaccine and Avridine and with RVFV vaccine alone is effective in reducing or preventing aerosol-acquired encephalitis, even though this route of priming is ineffective in preventing hepatitis. The late hepatitis, though unexpected, is consistent with the hypothesis that mucosal priming leads to systemic tolerance and reduced specific IgG and cell-mediated immunity. These data also show that s.c. priming with vaccine plus the immunomodulator Avridine results in mucosal, as well as systemic, protective immunity. Since vaccine alone is not effective in preventing the late encephalitis, results must be interpreted that the immunomodulator Avridine is responsible for producing a more diverse response that includes mucosal protection. In addition, these data confirm earlier studies that indicated the likely route of acquisition is via nasal mucosal transport into olfactory nerve tracts. Absence of secretory immunity in the nasal mucosa favors development of encephalitis, and the initial site of this encephalitis is in the olfactory bulbs, as determined by histopathology. In situ hybridization with a genetic probe for RVFV RNA will be carried out on unstained sections of brain and olfactory bulb to confirm the basis of these histopathological changes.

Mice that survived the virulent challenge, s.c. or aerosol, were bled. Plaque-reduction neutralization (PRN80) and ELISA titers of IgA, IgG, and IgM were determined. Data from a separate study are presented in Table 3. Although IgA is not normally measurable in serum, challenge by aerosol resulted in an increase in IgA over that produced after s.c. challenge. The effectiveness of s.c. injected vaccine plus Avridine in priming for a protective immune response, which included secretory IgA, is shown. In addition, there is an indication that aerosol challenge is capable of increasing specific IgG as well. The low IgM titers were expected since convalescent sera were used. All the sera had PRN80 titers >1:40 and the group given vaccine plus Avridine s.c. had titers >1:320.

Extramural collaboration was continued with Dr. Michael Potter (National Cancer Institute) on the ability of the immunomodulator Pristane to induce neoplastic transformation in cells of the immune system. Intraperitoneal inoculation of Pristane, the oil used in Freund's adjuvant, induced a foreign-body, granulomatous response in the peritoneal cavities of all mouse strains tested. Most BALB/c mice also develop a high frequency of peritoneal plasmacytomas; however, the BALB/cJ mouse, which differs from the other BALB/c strains at only a few genetic loci, is resistant to plasmacytoma induction. This study revealed additional features of the granulomatous response of BALB/c An Pt and BALB/cJ mice that correlated with their respective susceptibility or resistance to plasmacytoma induction after Pristane inoculation. Results indicate that the normal progression of the inflammatory response to mineral oil, from acute inflammation through chronic inflammation to healing, is qualitatively altered in BALB/c mice.

Foci of acute inflammation (the PMN pulsars) persist throughout the peritoneal response in both sublines and contribute acute inflammatory mediators and reactive products to the local microenvironment. Evidence that angiogenesis, stromal cell proliferation, and extramedullary myelopoisis are increased beyond what is needed for healing in the BALB/c An Pt mouse, suggests strong local influence of growth factors which might be offset by inhibitory factors in the BALB/cJ mice. Overextended growth of tissues involved in organization of the inflammatory site in the BALB/c An Pt mouse results in development of angiogenic polyps in which the earliest plasmacytomas develop.

The paucity of small lymphocytes in the polyps of BALB/c An Pt mice would not be obvious except when compared with the polyps of the BALB/cJ mice, since both are chronic inflammatory tissue by definition, and both contain some small lymphocytes. The difference is qualitative and quantitative; there are more small lymphocytes in BALB/cJ polyps, and they seem to have arrived there by a process analogous to lymphocyte recirculation. Peritoneal lesions in BALB/cJ mice contain dense aggregates of small lymphocytes in proximity with high endothelial venules (HEV) and interdigitating dendritic cells (IDC). Neither HEV nor IDC are present in Pristane-induced lesions in BALB/c An Pt mice. The significance of IDC and HEV is that they are structures normally found in the T-dependent cortex of lymph nodes. The IDC are believed to be the in vivo correlate of Ia-antigen-bearing dendritic cells which are potent stimulators of T-lymphocytes in vitro. HEV are critically important to lymphocyte recirculation. The specialized endothelial cells of HEV express, on their luminal surfaces, undefined determinants which are recognized by molecules on circulating lymphocytes, causing the lymphocytes to adhere and emigrate. Selective emigration of lymphocytes at HEV is fundamental to the continual repopulation of peripheral lymphatic tissues with lymphocytes which traffic throughout the body, and is the biological mechanism underlying clonal selection and antigen recognition in vivo.

Absence of trafficking small lymphocytes in the granulomatous polyps of the plasmacytoma-susceptible mice (while the resistant subline displays abundant small lymphocytes, HEV, and IDC) supports the proposition that small lymphocytes may somehow be involved in regulating the preneoplastic response of "transformed plasma cells." Factors that are responsible for the deficiency of small lymphocytes in Pristane-induced polyps of BALB/c An Pt mice are unknown, but differences in prostaglandin metabolism might be involved, since indomethacin treatment converts BALB/c An Pt polyps to a lymphocyte-rich phenotype and prevents plasmacytoma induction. While the BALB/c An Pt mouse may be excessive in proliferative activity, the BALB/cJ mouse has too much chronic inflammatory activity. BALB/cJ mice produce too much alphafetoprotein (AFP), which is an acute phase reactant that would be elevated in the blood of patients with arthritis or other diseases of inflammatory nature.

Although angiogenesis is known to be stimulated by tumors, the angiogenic process in this model precedes tumor induction by hundreds of days. The extensive angiogenic response to granulomatous inflammation that develops on the mesenteries of BALB/c An Pt, but not BALB/cJ, mice may contribute to the plasmacytomagenic environment. Increased vascularity of the peritoneal lesions in BALB/c An Pt mice may result in enhanced transport of inflammatory cells and oxygen, leading to increased production of clastogenic pro-oxidants by inflammatory cells. Dividing endothelial cells are known to release growth factors that specifically enhance division of plasma cells and hybridomas in vitro. Angiogenesis may mechanically contribute to the pedunculated shape of the lesions because of loss of continguity with the normal lymphatic microenvironments of the mesentery.

The resident population of cells in the polyps must be bathed in the metabolites and factors released by the predominant cell types, e.g., activated macrophages and polymorphonuclear leukocytes in various stages of degranulation. Clearance or detoxification of prostaglandins, interleukins, hematopoietic colony-stimulating factors, and mutagenic factors may be diminished by deposition of these factors on the droplets of Pristane. This might cause local concentrations to increase over time. Deficiences in lymphatics could also result in accumulations of growth factors and mutagens. However, the fact that acute inflammation and oil granulomas were present in the mesenteries of both BALB/c sublines favors genetic differences in response to the factors rather than differences in production or clearance in plasmacytoma induction.

Salient features of the Pristane-induced inflammatory response in BALB/c mice that correlate with plasmacytomagenesis are persistence of acute inflammation; angiogenesis; and other tissue proliferative phenomena, such as extramedullary myelopoiesis and mesothelial polyp formation; and deficiency of immunosurveillance of small lymphocytes. In contrast, the BALB/cJ subline differs from other BALB/c mice by exhibiting less proliferation activity and more infiltration with small lymphocytes.

Additional extramural collaboration was initiated with Drs. Beverly Mock and Carol Nacy (Walter Reed Army Institute of Research) on the immunomodulator-like effects induced in peripheral lymphatic tissues after footpad inoculation with *Leishmania Major*. Since these changes correlate with resistance and susceptibility to *Leishmania*-induced disease in genetically defined mouse strains, it becomes important to determine the mechanisms by which *Leishmania* alter the lymphatic microenvironments. This is in order to more rationally design immunomodulator therapies for humans who share similar genetic susceptibilities. Preliminary studies reveal that there is a reciprocal correlation between the ability of *Leishmania Major* to increase lymphocyte recirculation at high endothelial venules and resistance of these strains to *Leishmania* infestation. The effect seems to be dependent upon relative differences in induced mononuclear cell subclasses such as epithelioid cells and interdigitating dendritic cells.

<u>Viral Tropism</u> An in vitro system for evaluating viral tropisms was developed. Large quantities of biotinylated VEE virus (VEE-B) were produced by reacting virulent virus with the succinimide ester of biotin at graded concentrations. VEE-B retained viability and infectivity as demonstrated by the ability of the virus to produce cytopathic effects in tissue culture cells. Biotinylation did not reduce the number of PFU relative to starting concentrations of virus. VEE-B were applied to frozen sections of mouse head that included the nasal tubinates, cirbriform plate of the ethmoid bone, olfactory tracts, and brain. Selective binding of VEE-B was visualized as brown Avidin-horseradish peroxidase reaction product.

VEE-B adhered to the myelin sheath of the CNS and spinal cord axons. Nonmyelinated fibers above the molecular cell layers did not stain. Pyramidal and crossed pontine fibers bound the most virus, and association fibers of the cortex appeared to bind the least virus. Decreasing the amount of biotin on the virus decreased the staining intensity but increased the differential staining among the myelinated tracts. Blocking experiments showed the specificity of the receptor site as contrasted with non-specific adhesion of virus to fatty tissues.

The use of VEE-B on thin sections of tissue provided a rapid and simple method to screen tissues for receptor sites. Indentification of these sites will assist in determining the site of entry of virus into a target organ. Preliminary data indicate one of the sites of entry for VEE virus into the CNS is through myelinated axons. The binding of virus to myelineated axons is supported by findings in terminally ill animals that were exposed to VEE virus by s.c. injection. Virus, located by immunoperoxidase methods, was in the axons, in addition to the neurons of the CNS. Axon-VEE antigen levels were expecially dense in the myelinated nerve tracts below the molecular layer of the cerebellum.

<u>Mycotoxin Pathogenicity</u> Diacetoxyscirpenol (DAS) is a mycotoxin found in close association with the more frequently studied T-2 toxin. Like T-2, it is only slightly soluble in water, but is soluble in ethanol (ETOH) and dimethylsulfoxide (DMSO). Because DMSO and ETOH solutions have physical characteristics that are markedly different from aqueous solutions, the particle size of aerosols of the two solvents was determined in the animal exposure system. A 7-stage cascade impactor was used to sample aerosols of DMSO and ETOH containing sodium fluorescein. Airborne particles of ETOH in the exposure chamber had mean MMD of 0.63 μm (GSD 2.25), and particles of DMSO had 0.92 μm (GSD 2.83). Apparently the size is determined primarily by the geometry of the disseminator, rather than the viscosity of the solvent. The output of the nebulizer with DMSO, however, was considerably less than with ETOH.

In one study, groups of 12 ICR mice were exposed for selected time intervals to aerosols of 50 mg/ml of DAS dissolved in either DMSO or ETOH. Control groups of 12 mice received aerosols of only DMSO or ETOH. No animals died after exposure to DMSO-DAS, and control animals showed no visible response. With the exposure system used, doses greater than 0.625 mg/kg for mice could not be attained, even after 30-min periods. Lethality was observed when mice were exposed to aerosols of ETOH-DAS (Table 4). The amount of toxin required, however, was quite large. The 30-min exposure required about 300 mg of toxin.

Mice exposed to aerosols of 50 mg/ml of ETOH-DAS were killed after 4 and 24 h to determine blood and tissue distribution and to evaluate pathological alterations. Control mice were exposed to ETOH. Non-specific, tissue component interference prevented assay of toxin for distribution. Histologically, lymphoid necrosis and cellular degeneration were observed, as well as necrosis of the zona fasciculata of the adrenals. Adrenal pathology mimicked the changes induced with T-2 toxin (D. Creasia, Pathophysiology Division, personal communication). No changes were noted in the ETOH controls.

When F-344 rats were exposed to aerosols of DAS (50 mg/ml of diluent), all of the rats exposed to aerosols of ETOH-DAS succumbed, while 50% of those exposed to DMSO-DAS died (Table 5). Preliminary data suggest that the LD_{50} of ETOH-DAS for F-344 rats approximates 4.0 mg/kg (95% CL 2.9-6.5). The pathological alterations induced in rats by DAS were the same as occurred in mice; however, lesions were more severe.

PRESENTATIONS:

1. **Anderson A. O.** Lymphatic tissue: development, architecture of primary and secondary lymphatic tissues. Presented, Lecture #5, Immunology 404, University of Pennsylvania, Philadelphia, PA, 1 January 1985.

2. **Anderson, A. O.** Antigen localization lympoid cell migration, circulation, lodging, and alterations in lymphatic tissues during the course of an immune response. Presented, Lecture #6, Immunology 404, University of Pennsylvania, Philadelphia, PA, 4 February 1985.

3. **Anderson, A. O.** Organization of lymphoid tissue. Presented, Lecture #2, IMM 522M Cell Biology of Immunity and Inflammation Course, Foundation for Advanced Education in the Sciences, NIH, Bethesda, MD, 5 February 1985.

4. **Anderson, A. O.** Differences in the peritoneal oil granuloma response of Balb/cJ mice. Presented, NCI Workshop on Novel Immunological Responses of the Balb/c Mouse, NIH, Bethesda, MD, 12 March 1985.

5. **Anderson, A. O.** Viral tropisms in mucosal pathogenesis. Presented, USAMRIID Professional Staff Conference, Mar 1985.

6. **Anderson, A. O.** The role of angiogenic lymphatic microenvironments in the induction of autoimmunity. Presented, The Kennedy Institute of Rheumatology, London, England, 18 June 1985.

PUBLICATIONS:

1. **Rubin, D. H., M. J. Korstein, and A. O. Anderson.** 1985. Reovirus serotype-1 intestinal infection: a novel replicative cycle with ileal disease. *J. Virol.* 53:391-398.

2. **Potter, M., J. J. Wax, and A. O. Anderson.** 1985. Inhibition of plasmacytoma development in Balb/c mice by indomethacin. *J. Exp. Med.* 161:996-1012.

3. **Anderson, A. O., and D. H. Rubin.** 1985. Effect of Avridine on enteric antigen uptake and mucosal immunity to reovirus (1/Lang). *Adv. Exp. Med. Biol.* **186**:579-590.

4. **Anderson, A. O.** 1985. Multiple effects of immunologic adjuvants on lymphatic microenvironments. *Int. J. Immunotherapy* **2**:1-11.

5. **Anderson, A. O., T. J. MacDonald, and D. H. Rubin.** 1985. Effect of orally administered Avridine on enteric antigen uptake and mucosal immunity. *Int. J. Immunotherapy* **2**:12-21.

6. **Anderson, A. O., J. S. Wax, and M. Potter.** 1985. Differences in the peritoneal response to Pristane in Balb/c An and Balb/cJ mice. *Curr. Topics Microbiol. Immunol.* (In Press).

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8. **Kornstein, M. J., J. J. Brooks, A. O. Anderson, A. I. Levinson, R. P. Lisak, and B. Zweiman.** 1985. The thymus in myasthenia gravis: an immunohistologic study. *Adv. Exp. Med. Biol.* **186**:929-936.

Table 1. Protocol for contrasting intraduodenal with subcutaneous priming by using formaldehyde-inactivated Rift Valley fever virus vaccine (NDBR-103).

Vaccination				Challenge	
		Route			
Vaccine	Avridine	s.c.	i.d.	s.c.	Aerosol
+	+	+	-	+	-
+	+	+	-	-	+
+	-	+	-	+	-
+	-	+	-	+	-
-	+	+	-	+	-
-	+	+	-	-	+
+	+	-	+	+	-
+	+	-	+	-	+
+	-	-	+	+	-
+	-	-	+	+	-
-	+	-	+	+	-
-	+	-	+	-	+
-	-	-	-	+	-
-	-	-	-	-	+

Table 2. Histopathologic lesions in mice vaccinated with formaldehyde-inactivated RVFV vaccine and challenged with virulent virus.

Vaccination[a]	Challenge[b]	Histopathologic lesion[c]		
		Hepatitis	Encephalitis	Pneumonitis
Subcutaneous				
VA	s.c.	0	0	0
VA	A	14	71	21
V	s.c.	0	0	0
V	A	0	44	0
A	s.c.	33	0	0
A	A	50	25	25
Intraduodenal				
VA	s.c.	83	17	0
VA	A	71	0	0
V	s.c.	50	25	0
V	A	38	0	0
A	s.c.	-	-	-
A	A	100	20	0
Control				
-	s.c.	100	0	0
-	A	100	86	0

[a]V = vaccine, A = Avridine, VA = vaccine + Avridine
[b]A = aerosol exposure
[c]Percentage of challenged mice exhibiting histologic lesions

Table 3. RVFV-specific antibodies in vaccinated survivors after subcutaneous or aerosol challenge with virulent virus.

Vaccination[a]	Challenge[b]	Seropositive[c]/total tested		
		IgA	IgG	IgM
Subcutaneous				
VA	s.c.	1/6	6/6	1/6
VA	A	3/5	5/5	1/5
Intraduodenal				
VA	s.c.	0/4	3/5	1/5
VA	A	1/5	5/5	2/5

[a]VA = vaccine + Avridine
[b]A = aerosol exposure
[c]ELISA titer >1:20

Table 4. Response of ICR mice to aerosols of DAS dissolved in ethanol.

Exposure (min)	Estimated inhaled dose (mg/kg)	Dead/ total	LD_{50} (95% CL) (mg/kg)
30	3.3	9/12	
20	2.0	6/12	1.75
10	1.0	5/11	(1.24-2.77)
5	0.5	0/12	
2.5	0.25	0/12	

Table 5. Lethality in F-344 rats of aerosols of DAS dissolved in DMSO or ETOH.

Aerosol	Dead/ total	MDTD[a]
Ethanol (control)	0/8	-
DAS-ethanol[b]	8/8	1.1
DMSO (control)	0/8	-
DAS-DMSO[b]	4/8	1.41

[a]Mean days to death
[b]Contains 50 mg of DAS per ml of solvent, estimated dose of 0.5 mg/kg of the DAS-ethanol, and 0.2 mg/kg of the DAS-DMSO solutions

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SY
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AK	139
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Technology Development for Rapid Diagnosis and Identification of BW Agents

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE | EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE | d. AMOUNT
e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thou
85	0.8	169
86		

19. RESPONSIBLE DOD ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L
d. TELEPHONE NUMBER (include area code) 301-663-2833

20. PERFORMING ORGANIZATION
a. NAME Disease Assessment Division, USAMRIID
b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF PRINCIPAL INVESTIGATOR LeDuc, J W
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f. NAME OF ASSOCIATE INVESTIGATOR (if available) Knauert, F
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE FIC
MILITARY/CIVILIAN APPLICATION: M

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Immunology; (U) Lab Animals; (U) Rats; (U) Biotechnology; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop technology for rapid diagnosis and identification of BW agents in military clinical and environmental sphere. Field diagnosis will enhance the medical protection of US military personnel.

24. (U) To develop and refine state-of-the-art nucleic acid probes and other methods fo virus detection and identification.

25. (U) 8410-8509-We have developed a model nucleic acid hybridization assay for detecting Rift Valley fever virus (RVFV) in infected VERO cells and infected cell media using a cDNA probe which contains an insert of the middle-sized RNA (m-RNA) of this virus. With the model systems, we established the optimum conditions for preparing samples for detection and are able to detect complementary sequences in samples that contain as little as 25 pg of purified viral RNA, or 5 X 10^3 PFU. Using the model syste we are undertaking studies to determine whether or not it is possible to use this technology to distinguish various geographical isolates of RVFV as well as RVFV from oth members of the genus, *Phlebovirus*, and the family, Bunyaviridae. We have used the procedure developed in the model system to detect complementary sequences in RVFV-vaccin preparations, and modifications of this procedure to detect such sequences in human sera spiked with RVFV, in homogenates from aborted fetuses of pregnant ewes infected with RVF

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AK-139: Technology Development for Rapid Diagnosis and Identification of BW Agents

PRINCIPAL INVESTIGATOR: James W. LeDuc, LTC, MSC

ASSOCIATE INVESTIGATOR: Frederick Knauert, Ph.D.

Background:

Recent advances in recombinant DNA technology have led to the development of mixed-phase nucleic acid hybridization assays which have great promise as diagnosti tools (6,7,8,9,11). The basis of such assays is the ability of a probe containing sequences that are unique to a particular pathogen to hybridize with complementary sequences in a sample that has been immobilized onto a solid support, such as nitrocellulose paper, and thus serve to identify the presence of that organism in a sample (5). In practice, samples are filtered through a multi-well template under conditions that "fix" the nucleic acid as well as other cell constituents onto the nitrocellulose paper. The solid support containing up to 96 different samples, depending on the format used, is then exposed to the labeled probe under conditions that are compatible with hybridization. With this procedure, it is possible to analyze large numbers of samples in a relatively short period of time compared to liquid phase hybridization protocols previously used for this purpose, making it a realistic alternative method for detecting and identifying organisms on a large scale basis.

Recently, the collaborative efforts of Molecular Genetics, Inc., and the Viral Biology Group at USAMRIID have resulted in the cloning of sequences complementary t the M segment RNA segment of Rift Valley fever virus (RVFV) into bacterial plasmids (4), making available a probe that could serve to identify this virus in clinical and environmental samples. It has been our goal to use this probe to develop a mixed-phase hybridization assay for the detection of RVFV, and to evaluate its diagnostic usefulness relative to the methodologies currently used to detect and identify RVFV.

Summary:

Preliminary studies to evaluate two methods for preparing samples for hybridization showed that the "cytodot" protocol (12) in conjunction with the "slot-blot" (10) filtration format was the best method for preparing samples for the detection of complementary RVFV-RNA sequences. This work led to the development of a model assay system for detecting purified RVFV-RNA samples, RVFV-RNA from samples of infected cells, and RVFV-RNA from samples of infectious cell media. By this model system, we were able to detect RVFV-specific sequences routinely in 0.025-ng samples of purified RNP-RNA, in 8.0 ng samples of purified RNA containing all species of viral RNA in at least a hundredfold excess of cellular RNA, in samples oi infected cells which yielded 1 - 2 x 10^4 PFU, and in samples of infectious cell media containing 4 x 10^4 PFU of virus. We applied the information gained from the development of the model system to the detection of viral sequences in non-model, clinically and environmentally relevant samples. By using the standard protocol

developed for the model system, we were able to detect RVFV sequences in inactivated vaccine preparations and samples of aerosolized virus collected post-aerosol exposure. By modifying the standard protocol to decrease excessive amounts of cellular components which interfere with proper filtration and compromise optimum hybridization reactions, we have developed assays for detecting RVFV-RNA sequences in human serum spiked with RVFV, in viremic sheep serum, and in homogenates of RVFV-infected mosquitoes. We have also been able to detect RVFV-sequences in tissue homogenates from aborted fetuses of RVFV-infected ewes. These experiments were carried out according to a standard protocol and suffered from the problems associated with excessive cellular constituents and poor filtration. Because such samples were in limited supply, we have not been able to try the modified procedures on these samples. When equivalent samples become available, the experiments will be repeated with the modified protocols.

<u>Progress:</u>

Initial experiments were carried out to determine whether the "cytodot" procedure of White and Bancroft (12) or the "quick-dot" procedure of Bresser et al. (1,2,3) was better for preparing samples for detecting complementary RVFV-sequences in a mixed-phase hybridization assay. At the same time we compared the efficacy of two filtration formats, the standard dot-blot format, and a more recent modification, the slot-blot format (10), to determine whether either of these formats offered any advantage over the other.

These studies showed that with the dot-blot filtration format, both of the sample preparation protocols were approximately equivalent for preparing samples of infected cells, enabling us to detect complementary sequences in samples that yielded 1-2 x 10^4 PFU of RVFV. However, the "cytodot" procedure was superior for detecting sequences in samples of infectious cell media and samples of RNA purified from ribonuclear particles (RNP). In the former case, the "cytodot" procedure was able to detect complementary sequences in media samples containing 4 x 10^4 PFU, whereas with the "quick-dot" method, we were unable to detect viral sequences in samples containing 3 x 10^5 PFU. The "cytodot" procedure was approximately 60 times more sensitive than the "quick-dot" procedure for detecting RNP-RNA, enabling us to detect complementary sequences in a 0.1-ng sample of purified RNP-RNA, versus 6.25 ng for the "quick-dot" procedure. We were unable to detect RVFV-RNA in samples of RNA that had been purified by centrifugation through a 20-40% cesium chloride gradient by either of these preparation protocols with the dot-blot filtration format. This pelleted fraction of RNA contains all the various RVFV-RNA species that are produced during the course of an infection, as well as at least a hundredfold excess of cellular RNA, primarily ribosmal RNA. However, we were able to increase the sensitivity of the assay by incorporating the slot-blot filtration format into the procedure. By using the "cytodot" procedure in conjunction with the slot-blot format, we were able to detect RVFV-specific sequences in an 8.0-ng sample of purified pelleted RNA. The "quick-dot" procedure is not compatible with the slot-blot filtration format so it was not possible to test that combination.

We also tested the cytodot/slot-blot combination for preparing samples of purified RNP-RNA, infected cells, and infectious cell media, and found that, although the incorporation did not significantly improve the sensitivity of the assay in quantitative terms, it did improve it qualitatively. While the endpoint dilution of detection was about the same in these samples for both filtration formats, the signal from the slot-blot format was qualitatively superior. A sharper, more easily read signal made it easier for us to discriminate weak positive signals from signals resulting from non-specific binding of the probe to cellular components. Based on these findings, we adopted the "cytodot" preparation procedure with the slot-dot filtration format as our standard protocol.

Having established the above procedure as a standard, we proceeded to test clinically and environmentally relevant samples to see if this assay was applicable to non-ideal model situations. One of the samples tested was inactivated-RVFV vaccine lots. The objectives of this study were twofold: first, to determine whether we could use hybridization as an *in vitro* assay for vaccine efficacy. The rationale behind this objective was that steps in the production process that were deleterious to the immunogenicity of the vaccine might also be deleterious to the nucleic acid of the virus and might be reflected in the nucleic acid content of these preparations as measured by hybridization. The second objective was to directly compare the relative sensitivity of the hybridization procedure with an ELISA procedure used to evaluate the antigen content of these vaccine preparations. These studies showed that there was no correlation between vaccine efficacy and nucleic acid content; i.e., there was no significant difference in the intensity of the hybridization signals, or the endpoint dilution detection limits generated from serially diluted samples of effective and ineffective vaccine lots. Based on the endpoint dilution limits of detection of these samples, we were able to determine that the hybridization assay was at least as sensitive as the ELISA procedure, and depending on how long one wanted to spend exposing the hybridized nitrocellulose sheet to X ray film, could be 4 to 8 times more sensitive.

Other samples in which we successfully detected RVFV were aerosol samples provided to us by Cmdr. O. Wood of the Airborne Disease Division. The specimens were collected post-aerosol-exposure and were used to calculate the per mouse exposure dose. These results were particularly gratifying in that, according to Dr. Wood, this is the first instance that a rapid diagnosis technique was used to detect virus in such samples.

When we tested a number of other potentially relevant samples, such as viremic serum, tissue homogenates, or mosquito homogenates with the standard protocol, we encountered a filtration problem. These samples filtered either very slowly or not at all through the nitrocellulose paper. We have evidence that the problem is related to excessive amounts of soluble macromolecules, particularly proteins and DNA, in these samples that adsorb to and eventually clog the nitrocellulose paper and block filtration. This has a number of adverse effects on the hybridization signal. When samples are incompletely filtered, only a portion of the relevant sequences come in contact with and are immobolized onto the nitrocellulose paper. Thus, only a fraction of these sequences are available for hybridization. Since proteins, DNA, RNA, and other macromolecular constituents adsorb to nitrocellulose under standard conditions, virus-specific RNA is at a numerical disadvantage when competing for a limited number of binding sites in excessive amounts of non-specific material. Furthermore, the hybridization probe non-specifically binds to a limited degree to nucleic acids and proteins. Under normal conditions, with the use of appropriate controls, this binding is insignificant and easily distinguishable from specific hybridization reactions. However, in the presence of excessive amounts of protein and non-virus nucleic acids, the non-specific signal increases, and since the amount of non-specific material is unknown, it is difficult to select the appropriate control to discriminate weak positive signals from strong, non-specific signals. Finally, when the filtration template becomes clogged, samples tend to spread beyond template boundaries, resulting in an irregularly shaped, diffuse signal that is less than optimal for analysis. To alleviate these problems, we have tried a number of modifications to the standard protocol to improve the filterability of the samples and, in turn, the quality of the hybridization signal.

By pretreating human serum samples with the protease, proteinase K, we were able to detect RVFV-specific signals in samples that had a virus titer of 10^4 PFU/ml. Because the supply of authentic human viremic blood samples is limited, we created artificial viremic samples by spiking pooled human sera with a known quantity of virus. Now that we have established optimum conditions for testing human serum samples, we are in the process of evaluating the effect of preincubating the virus with RVFV-specific antibody prior to immobilizing the samples on nitrocellulose, to test the effect of immune complexes on the assay. Once these conditions and effects are optimum, we feel that we will be in the position to test authentic viremic sera with a reasonable chance of success.

We have also been able to use the proteinase K-modified procedure to detect RVFV in authentic viremic sheep serum samples. The samples, provided by Major John Morrill of our Division, were collected on consecutive days following inoculation of sheep. The virus titers were established by a standard plaque assay. We were able to clearly detect virus-specific sequences on day 2, 3, and 4 post-inoculation, where there were 5.64, 4.45, and 2.05 logs of virus present in the samples. We were unable to detect virus on day 1 post-inoculation when there were 2.13 logs of virus present. We do not know the basis of discrepancy between the day 1 sample (2.13 logs of virus) and the day 4 sample (2.05 logs of virus). One possibility is that by day 4, RVFV-specific antibodies were present and were affecting the virus titer. We also saw weak signals on days 6 and 7 post-inoculation, when virus was undetectable by plaque assay. Again the presence of RVFV-specific antibody may be a reasonable explanation for this observation, but at present, we do not have any evidence to support this hypothesis. One problem that confounds the interpretation is that the proteinase K treatment is not as an effective remedy for correcting the filtration problem in sheep sera as it was for human sera. Therefore, the weak signals we observed may be due to non-specific binding of the probe. It is unclear from these experiments whether the differences in susceptibility of these two serum types to proteinase K pretreatment is a consequence of intrinsic differences in these sera, of the way the serum was prepared, of some unknown factor(s), or of a combination of these factors. Experiments are underway to improve the filterability of the sheep serum samples and to try to answer some of the questions regarding the possible impact of immune complexes on the interpretation of these results.

We have also been able to use the hybridization assay to detect RVFV in infected mosquito homogenates provided by Dr. M. Turell of our Division. In this case it was necessary to pretreat the samples with DNase I. We established the conditions for DNase pretreatment by using pooled mosquito homogenates spiked with known amounts of virus. With this system, we were able to detect virus in pools with a virus titer of 1×10^4 PFU/ml. Interestingly, we found that if we combined the proteinase K pretreatment with the DNase I pretreatment, the resulting signal was diminished. We were also able to detect virus successfully in homogenates prepared from individually infected mosquitoes, where titers were 10^4-10^5 PFU virus. We are currently in the process of testing this procedure to determine the infectivity status of a coded group of mosquito homogenates with high, medium, and low virus titers, as well as uninfected controls.

With the standard, unmodified procedure, we have also been able to detect RVFV-sequences in sheep tissue homogenates provided by Major Morrill. These tissue samples were prepared from aborted fetuses of RVFV-infected ewes. With the standard protocol we were able to detect viral sequences clearly in liver, heart, eye, and probably thymus tissue homogenates. The samples were also positive by either ELISA and/or plaque assays. However, we were not able to detect such sequences in cerebrum samples, which usually have the highest infectivity titers of all samples. The reasons for this are unknown and under investigation. As with the serum sample and mosquito homogenate studies, these experiments suffered from the problems associated with poor filterability. Because these samples were in limited supply, we have not been able to test the protease and nuclease modifications carried out on the other samples. Such experiments will be performed when equivalent samples become available.

PRESENTATIONS:

1. **Knauert, F. K., M. D. Parker, and J. M. Dalrymple.** Use of cDNA probes to detect Rift Valley fever virus. Presented, Annual Meeting of the American Society for Virology. 21-25 July, 1985, University of New Mexico, Albuquerque, NM.

PUBLICATIONS: None

LITERATURE CITED

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2. **Bresser, J., J. Doering, and D. Gillespie.** 1983. Quick-dot: selective mRNA or DNA immobilization from whole cells. *DNA* **2**:243-254.

3. **Bresser, J., H. R. Hubbell, and D. Gillespie.** 1983. Biological activity of mRNA immobilized on nitrocellulose in NaI. *Proc. Natl. Acad. Sci.* U.S.A. **80**:6523-6527.

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10. **Tisty, T., P. C. Brown, R. Johnston, and R. T. Schimke.** 1982. Enhanced frequency of generation of methotrexate resistance and gene amplification in cultured mouse and hamster cell lines, pp. 231-238. *In* R.Y. Schimke (ed.), *Gene amplifications.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

11. **Vertanen, M., A-C Syvanen, J. Oram, H. Soderlund, and M. Ranki.** 1984. Cytomegalovirus in urine: detection of viral DNA by sandwich hybridization. *J. Clin. Microbiol.* 20:1083-1088.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA 302653	2. DATE OF SUMMARY: 85 10 01	REPORT CONTROL SYMBOL: DD-DR&E(AR) 636
3. DATE PREV SUM'RY: 84 10 01	4. KIND OF SUMMARY: H. TERM	5. SUMMARY SCTY: U	6. WORK SECURITY: U	7. REGRADING	8. DISB'N INSTR'N: CX	9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AL	140
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) (U) Exploratory Research For Protection Against Hantaan Virus

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	85 10	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	3.6	196
86	0.0	0

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Dalrymple, J M
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7241
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Schmaljohn, C S
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available) LeDuc, J W

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Prophylaxis; (U) Chemotherapy; (U) Vaccines; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To investigate molecular and biological properties of viruses in the newly-described *Hantavirus* genus of Bunyaviridae and identify viral characteristics useful for diagnosis and prophylaxis of associated diseases.

24. (U) Examine the nucleic acid of prototype Hantaan virus by using recombinant DNA techniques to clone the genes which encode the major virus antigens. Determine the molecular characteristics of the viral structural proteins and carbohydrate residues, and examine cross-reactivities of Hantavirus proteins. Investigate methodologies useful for development of a rapid viral diagnostic test. Describe the natural epidemiology of Hantaviruses.

25. (U) The medium (M) and small (S) genome segments of Hantaan virus were cloned and partially sequenced. The S segment was shown to code for the nucleocapsid protein. No subgenomic messages could be detected and data suggested that the M segment encodes the two viral envelope glycoproteins. Examination of the structural proteins of several Hantaviruses revealed three different profiles which correlate with antigenic groups. High mannose, N-linked carbohydrate residues which were detected on both envelope glycoproteins were found to be important for efficient viral replication. Methods for preparation of large quantites of inactivated Hantaan antigen for use in rapid virus diagnostic tests were investigated. Vertical transmission of a Hantavirus in rats was found to be abrogated by Caesarian delivery and foster nursing of offspring.

This research will be part of W.U. 871AD in FY 86.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AL-140: Exploratory Research for Protection Against Hantaan Virus

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ASSOCIATE INVESTIGATORS: Connie S. Schmaljohn, Ph.D.
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John D. White, Ph.D.

Background:

Hantaan (HTN) virus is the presumed etiologic agent of Korean hemorrhagic fever. It has a three-segmented, single-stranded RNA genome of negative polarity and has been proposed as the prototype of a new genus of Bunyaviridae, the *Hantavirus* genus (3,4). Viruses serologically related to HTN are believed to cause a variety of clinically similar diseases which are collectively termed hemorrhagic fever with renal syndrome (HFRS). In addition to viruses implicated in HFRS, many hantaviruses have now been isolated from rodents in geographic regions with no known associated disease (1,2). The ubiquity of these viruses in nature and their transmission via aerosolization rather than an arthropod vector, impart a unique disease threat to these agents. We have used both molecular and epidemiological methods to examine the natural properties of these viruses with the intent of defining specific characteristics which will lead toward more effective diagnosis and disease control.

Summary:

The structural proteins of eight representative hantaviruses were compared by cross-immune precipitation with hyperimmune rat antisera. Three distinct profiles emerged which were found to correlate with previously defined antigenic groups. Examination of the carbohydrate components of the envelope glycoproteins of HTN revealed that attached residues were mostly, but not entirely, of the high mannose type, and all appeared to be N-linked. Disruption of normal glycosylation processes with the antibiotic tunicamycin or with the ionophore monensin demonstrated that glycosylation was essential for infectious virus production.

Recombinant DNA (cDNA) clones of the middle (M) and small (S) genome segments of HTN virus were produced in plasmid pBR322 and replicated in *E. coli* HB101. Defined regions were subcloned into bacteriophage M13 and sequence analysis was performed by the chain termination method. Double-stranded and strand-specific cDNA were used to probe infected cell lysates resulting in the identification of HTN messenger RNA species. The nucleocapsid protein of HTN was translated in vitro, immune-precipitated with monoclonal antibodies, and demonstrated to originate from the S genome segment. No subgenomic M or S RNA species were detected, suggesting that each of these genome segments represented a monocistronic message encoding both envelope glycoproteins and the nucleocapsid protein, respectively.

Rapid virus diagnostic tests were investigated and preliminary parameters established for optimal production of large quantities of HTN antigen by treatment with beta-propiolactone. An IgM capture enzyme immunoassay was employed for experimental diagnosis of HFRS cases which occurred in Greece, and attempts to isolate this presumed, new, disease-causing hantavirus were pursued.

Serological screening of certified rat cell lines available from the American Type Culture Collection indicated that these lines were free of hantavirus antigens. Antibody screening was performed on sera samples submitted from various regions of the world and confirmatory diagnoses were made.

The vertical transmission of a hantavirus in rats was found to be abrogated by caesarian delivery and foster nursing.

Epidemiological investigation of Puumala virus (the etiologic agent of nephropathia epidemica) in Sweden resulted in definition of endemic disease areas and geographic boundaries of rodent hosts.

Progress:

Representative hantaviruses could be placed into one of three antigenic groups by radioimmune assay cross-reactivity (3). These groups included: (1) prototype HTN and the human isolate Lee; (2) rat isolates from Korea, Japan and the United States; and (3) a *Clethrionomys* isolate from Finland and a *Microtus* isolate from the United States. The more specific plaque-reduction neutralization test further distinguished the isolates in group 3 into separate antigenic categories (Figure 1). Examination of the structural proteins of these viruses demonstrated that unique profiles exist for each of the antigenic groups (Figure 2). Each isolate was shown to have a nucleocapsid protein (N) and two envelope glycoproteins (G1 and G2). Approximate molecular weights of N, G1 and G2 for each of three antigenic groups were:

Antigenic Group	Mr		
	N	G1	G2
1	50,000	69,000	54,000
2	50,000	76,000	55,000
3	53,000	64,000	58,000

We examined the cross-reactivity of the proteins of several hantaviruses which represented these antigenic groups by immune precipitation of viral proteins from infected cell lysates by using hyperimmune, polyclonal rat sera prepared by infection with individual isolates. Identification of cross-reactive proteins of isolates is important both for diagnostic and immunization purposes. The most cross-reactive protein should be best for the development of screening assays and the least cross-reactive for type-specific identification of the virus. Similarly, definition of conserved epitopes or antigenic determinants shared by all viruses would suggest possible protection of people from a variety of forms of HFRS following immunization with a single virus isolate. We found that the three structural proteins of HTN and Lee were precipitated almost equally by homologous immune sera and antisera to each other, and could be precipitated, but to a lesser extent, by antisera directed against the rat viruses. Antisera to the four rat viruses tested precipitated structural proteins of all rat viruses but reacted less well with HTN and Lee and demonstrated the greatest cross-reactivity with the nucleocapsid protein. Antisera directed against the *Clethrionomys* or *Microtus* isolates did not precipitate any polypeptides of other isolates, and only the nucleocapsid protein of these two viruses could be precipitated with heterologous sera. These data indicate that the nucleocapsid proteins of hantaviruses are more cross-reactive than the envelope proteins, which suggests that these proteins would be valuable diagnostic screening reagents. Conversely, the envelope proteins appear more valuable for type-specific diagnosis. Preliminary monoclonal antibody studies have shown that epitopes are also conserved on the glycoproteins of several of these viruses. As expected, the only neutralizing monoclone currently available to us is directed against an envelope protein, suggesting that these proteins are important for immunity. Additional monoclonal antibodies will be required to determine exactly which epitopes are conserved among hantaviruses and the role they play in immunity or protection.

In addition to structural protein characteristics, the carbohydrate components of the envelope glycoproteins may be involved in correct assembly, transport, and release of infectious viruses. To determine the amount and type of sugar present on G1 and G2 of representative hantaviruses, two different enzymes capable of cleaving sugars from a protein backbone were used. Both endoglycosidase H, which cleaves only high mannose residues, and endoglycosidase F, which cleaves high mannose and complex sugars, resulted in a decrease in molecular weight of ^{35}S-methionine labeled G1 and G2 by approximately 7,000 and 3,000, respectively. Radiolabeling of the carbohydrate residues with ^{3}H-mannose and similar cleavage revealed complete removal of the radiolabel by endoglycosidase F but not endoglycosidase H, indicating that most, but not all, of the glycosylation was of the high-mannose type.

To determine what effects disruption of the normal glycosylation process would have on production and release of infectious virus particles, infected cells were treated with several concentrations of two different drugs which interfere with glycosylation. The antibiotic tunicamycin directly prevents N-linked glycosylation and the ionophore monensin interferes with transport mechanisms of the Golgi; an important step in Bunyaviridae maturation. Both drugs were found to significantly reduce infectious virus intracellularly and yields in cell supernatants. Not only was the yield of infectious virus reduced, but particle formation in general was inhibited. At the molecular level, differential effects of the drug were observed. In the presence of monensin, reduced quantities of protein were observed as compared to untreated cultures, yet polypeptides which were synthesized were electrophoretically indistinguishable from untreated viral proteins. Tunicamycin, however, demonstrated little effect on the nucleocapsid protein, but appeared to severely inhibit synthesis of envelope proteins. Tunicamycin treatment in the presence of protease inhibitors suggested that the envelope proteins of HTN were synthesized normally but were more susceptible to proteolytic digestion in their unglycosylated form. From these studies, it is apparent that glycosylation of HTN envelope proteins is important both quantitatively and qualitatively for production of normal viral proteins and infectious virus particles.

Previously we demonstrated that the genetic characteristics of HTN suggest classification in a separate genus in the Bunyaviridae family of viruses (3,4). Although the replication strategies of viruses in the existing four genera of Bunyaviridae vary widely, all viruses examined to date have been demonstrated to utilize the small (S) genome segment to encode the nucleocapsid protein and the medium (M) segment for the envelope glycoproteins. To examine the coding strategy of HTN virus, messenger RNAs were isolated and translated into proteins in cell-free systems. A message the size of genomic S produced a polypeptide which could be immune-precipitated with monoclonal antibodies directed against authentic HTN nucleocapsid protein, suggesting that the S segment of HTN, like that of other Bunyaviridae, codes for the nucleocapsid protein. HTN envelope glycoproteins were not translated in vitro, even in systems with added microsomal membranes or by polysome runoff translation. To identify additional, potential messenger RNA species, infected cell lysates were probed with double-stranded or strand-specific cDNA probes.

The M and S genome segments of HTN virus were transcribed to cDNA with AMV reverse transcriptase and synthetic oligonucleotides complimentary to the 3' ends of virion RNA to prime first strand synthesis. Usual cloning procedures were employed and the double-stranded HTN cDNA was inserted into the PSTl site of the plasmid pBR322 and replicated in *E. coli* HB101 (Figure 3).
Recombinant clones were identified and characterized. Partial restriction maps of the largest M and S inserts obtained are displayed in Figure 4. These clones appeared to represent most or all of the M and S genome segments.

Radiolabeled, double-stranded hybridization probes were produced by nick translation of cloned DNA. Single-strand probes were prepared by subcloning defined genome regions into the single-strand bacteriophage M13, and subsequently synthesizing radiolabeled cDNA corresponding to the insert. DNA was force-cloned in both orientations so that single-stranded probes were obtained which were both virion genome-sense and anti-genome (message sense). Probes were used to hybridize to viral RNA extracted from infected cell lysates or virions which had been size-fractionated by methyl-mercury agarose gel electrophoresis and transferred to nylon membranes by electroblotting. All probes detected only genomic length M and S RNA in infected cell lysates and virions. These data suggest that subgenomic messages are not used to code for viral proteins, but rather that genomic length S codes for the nucleocapsid protein and genomic length M for the two envelope glycoproteins. Furthermore, it appeared that both virion and anti-virion sense RNA was encapsidated and released in virus particles. Whether this has any significance for viral replication is unclear.

The ultimate definition of viral coding strategy will be obtained from examination of the sequence organization of the M and S segments, especially the open reading frames used to code for viral proteins. Current sequence data obtained for the M segment indicate that protein synthesis could be initiated at either of two in-frame, initiation codons at nucleotide positions 40 and 64. A single open reading frame continues for at least 900 bases. Approximately 1400 bases of M (40%) and 1500 bases of S (90%) have now been sequenced and it is anticipated that the complete nucleotide sequences of both M and S will soon be determined. The successful recombinant cloning of the M and S genome segments of HTN virus can soon be exploited for the development of extremely sensitive probes for infected cells and tissues and should also provide the necessary tools for further delineation of viral replication mechanisms.

A rapid diagnostic assay for hantaviruses was investigated. Large volumes of antigen were prepared for each of the hantaviruses known to cause human disease: prototype Hantaan; Urban Rat (Seoul virus); and Puumala viruses. Antigens were inactivated following standard treatment with beta propiolactone (BPL); however, residual live virus was detected when treated antigens were cocultivated with Vero E-6 cells. Doubling the amount of BPL or addition of gamma radiation treatment appeared to completely inactivate the virus. Tests are still in progress to determine the optimum inactivation procedure which will retain maximum antigenicity/reactivity in the diagnostic test.

Collaborative studies with Dr. A. Antoniades (USAMRDC contractor) in Greece have confirmed our earlier observations of a severe form of HFRS in Greece. A total of 20 hospitalized, serologically confirmed cases of HFRS have now been diagnosed. Of these, 12 (60%) suffered a severe clinical course, and renal or peritoneal dialysis was required in 8 (40%) cases. Three (15%) patients ultimately succumbed. Serological and epidemiological studies have confirmed that the disease is not due to Puumala virus infection, as previously suspected. Patients have little or no antibody to Puumala virus by plaque-reduction neutralization testing (PRNT), and the natural rodent host, *C. glareolus* was absent from areas where patients were likely to have been infected. Patients' sera reacted strongly by PRNT with both prototype Hantaan virus and Seoul virus; however, both *Apodemus agrarius* and domestic rats (*Rattus*), natural hosts for these viruses, have been excluded as potential sources of infection for several patients. Our current impression is that a new hantavirus is responsible for this disease. Attempts to isolate this agent remain in progress.

Acute and early convalescent human sera from hospitalized patients infected in Greece were examined in an IgM capture enzyme immunoassay (EIA). Specific anti-hantavirus IgM antibody was detected, frequently as strongly positive reactions, in most, but not all sera examined from patients on hospital admission. A single day 5 serum was positive, a day 7 serum was negative, all four day 8 sera were positive, and 4 of 5 sera from days 9 or 10 were positive. These results suggest that this test holds promise for rapid diagnosis of hospitalized HFRS patients.

Several sera have been submitted from various areas of the world for confirmatory testing for antibody to hantaviruses. Approximately 30 human sera from Sudan with reported reactivity to Hantaan virus by IFA were retested by both IFA and PRNT using Hantaan, Seoul, and Puumala viruses. All sera were negative. Five rat and five human sera from Sicily were similary tested and also found negative. Acute and convalescent sera from a patient seen in France all had high-titer antibody to Puumala virus.

Preliminary experiments were conducted to determine if Seoul virus was passed from infected-rat mothers to their progeny. This question is relevant to determining the methods of virus maintenance in free-living rodents as well as practical application as to possible ways to salvage important strains of laboratory rats which may be chronically infected with hantaviruses. Female rats were infected with Seoul virus at various times prior to pregnancy or delivery. Offspring was either caesarean-delivered, passed through a disinfectant wash, then foster-nursed on uninfected mothers or allowed to progress through natural delivery. Preliminary results indicate that caesarean delivered, foster-reared young remained free of Seoul virus infection while those naturally delivered were infected. This experiment will be repeated during FY 86 to ensure the accuracy of our findings; however, these preliminary results are clearly promising.

Chronic infection of wild and laboratory rats with hantaviruses suggests that cell lines of rat-origin may also harbor hantaviruses. To examine this possibility, ampules of all rat-origin cell lines and all rat-mouse hybridomas housed at the American Type Culture Collection were examined for the presence of hantaviral antigen by immunofluorescent antibody assay. A total of 40 rat-origin, continuous cell lines and 32 antibody secreting lines were examined and found free of hantaviral antigen.

Results of a three-year collaborative study on the epidemiology of nephropathia epidemica in Sweden were summarized. The study involved three aspects: a countrywide human serosurvey for antibody to Puumala virus, cause of nephropathia epidemica; a countrywide small-mammal survey in which both Puumala virus antigen and antibody prevalence rates were determined; and a determination of the incidence of nephropathia during the 1984-85 transmission season. Previous studies had yielded an isolate of Puumala virus from voles captured in Sweden, and this agent was used in all serological testing except a portion of the 1984-85 cases which were serologically confirmed at another laboratory using prototype Hantaan virus. Serosurvey results showed antibody to Puumala virus most abundant among populations residing in the northern two-thirds of Sweden, and lowest or absent in southern Sweden. Incidence rates of nephropathia epidemica during 1984-85, a peak vole year, showed highest rates in two northern counties, AC and Y counties, with rates of >20 cases/100,000 population based on December 1983 census populations. Incidence rates of 10-19/100,000 were found for BD and Z counties, 1-9/100,000 for S, W. and X counties, and less than 1/100,000 for the remaining counties, all of which are in southern Sweden. Small-mammal collections found Puumala virus antigen and antibody abundant only among *Clethrionomys glareolus* and rarely in other species tested. While *C. glareolus* was commonly captured throughout Sweden, Puumala virus antigen and antibody were only found in northern populations. The boundary which separates endemic and nonendemic areas of nephropathia epidemica appears to correspond with the limes norrlandicus, a biogeographical boundary which separates the northern boreal zone from the southern nemoral zone. This line also demarcates the cyclic *C. glareolus* population of the north from noncyclic southern populations. Additional studies of northern and southern vole populations are in progress to determine the role of Puumala virus infection in vole cyclicity. The highly endemic regions of AC and Y counties which have been identified will now offer us test populations for evaluation of rapid diagnostic assays and, perhaps, improved therapeutic interventions.

PRESENTATIONS:

1. **Schmaljohn, C. S., S. Hasty, and J. M. Dalrymple.** Molecular and antigenic comparison of eight viruses in the newly proposed Hantavirus genus of Bunyaviridae. Presented, XI International Congress for Tropical Medicine and Malaria, Calgary, Alberta, Canada, October 1984.

2. **Schmaljohn, C. S., S. Hasty, and J. M. Dalrymple.** The antigenic and structural protein relationships of Hantaviruses. Presented, 33rd Annual Meeting of the American Society of Tropical Medicine and Hygiene, Baltimore, December 1984.

3. **Schmaljohn, C. S.** The molecular biology of Hantaan virus. Presented, Virology Professional Staff Conference, USAMRIID, May 1985.

4. **Schmaljohn, C. S., and J. M. Dalrymple.** Identification of Hantaan virus messenger RNA with recombinant DNA clones to the M and S genome segments. Presented, Annual Meeting of the American Society for Virology, Albuqerque, July 21-25, 1985.

5. **Schmaljohn, C. S.** The replication of Hantaan Virus. Presented, Oxford University, Oxford, England, 12 September 1985.

6. **Schmaljohn, C. S., G. B. Jennings, and J. M. Dalrymple.** Identification of Hantaan virus messenger RNA species. Presented, at The Biology of Negative Strand Viruses Meeting, Robinson College, Cambridge, England, 15-20 September 1985.

7. **LeDuc, J. W.** Epidemiology of Hantaan and related viruses. Annual Meeting, American Association of Laboratory Animal Scientists, Cincinnati, Ohio, November 1984.

8. **LeDuc, J. W.** Epidemic Report: severe hemorrhagic fever with renal syndrome in Greece. American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

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1. **Schmaljohn, C. S., S. E. Hasty, J. M. Dalrymple, J. W. LeDuc, H. W. Lee, C. H. von Bonsdorff, M. Brummer-Korvenkontio, A. Vaheri, T. F. Tsai, H. L. Regnery, D. Goldgaber, and P. W. Lee.** 1985. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. *Science* **227**:1041-44.

2. **Collett, M. S., A. F. Purchio, K. Keegan, S. Frazier, W. Hays, D. K. Anderson, M. D. Parker, C. S. Schmaljohn, J. Schmidt, and J. M. Dalrymple.** 1985. Complete nucleotide sequence of the M RNA segment of Rift Valley fever virus. *Virology* **144**:228-45.

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4. **LeDuc, J. W., G. A. Smith, and K. M. Johnson.** 1984. Hantaan-like viruses from domestic rats captured in the United States. *Am. J. Trop. Med. Hyg.* **33**:992-98.

5. **LeDuc, J. W., G. A. Smith, F. P. Pinheiro, P. F. C. Vasconcelos, E. S. T Rosa, and J. I. Maiztegui.** 1985. Isolation of a Hantaan-like virus from Brazilian rats and serologic evidence of its widespread distribution in South America. *Am. J. Trop. Med. Hyg.* **34**:810-15.

6. **Childs, J. E., G. W. Korch, G. A. Smith, A. D. Terry, and J. W. LeDuc.** 1985. Geographic distribution and age related prevalence of antibody to Hantaan-like virus in rat populations of Baltimore. *Am. J. Trop. Med. Hyg.* **34**:385-387.

7. **LeDuc, J. W., G. A. Smith, M. Macy, and R. J. Hay.** Certified cell lines of rat origin appear free of Hantavirus infection. *J. Infect. Dis.* (In press).

8. **LeDuc, J. W., G. A. Smith, J. E. Childs, F. P. Pinheiro, J. I. Maiztegui, B. Niklasson, A. Antoniades, D. M. Robinson, M. Khin, K. F. Shortridge, M. T. Wooster, M. R. Elwell, P. L. T. Ilbery, D. Koech, E. Rosa, T. Salbe, and L. Rosen.** Global survey of antibody to Hantaan related viruses among peridomestic rodents. *Bull. WHO.* (In press).

9. **LeDuc, J. W., and K. M. Johnson.** Hantaan virus: causative agent of Korean hemorrhagic fever. manual of microbiologic monitoring of laboratory animals. *In* A. M. Allen (Ed.). (In press).

10. **LeDuc, J. W., G. A. Smith, K. M. Johnson, F. P. Pinheiro, and J. I. Maiztegui.** Urban rats as hosts of Hantaan-like viruses in the Americas. *In* A. Diwan (Ed.), Korean hemorrhagic fever and related viruses. (In press).

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15. **LeDuc, J. W.** Epidemiology of Hantaan and related viruses. 1985. *Lab. Animal Sci.* (Submitted).

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2. **LeDuc, J. W., G. A. Smith, F. P. Pinheiro, P. F. C. Vasconcelos, E. S. T. Rosa, and J. I. Maiztegui.** 1985. Isolation of Hantaan-like virus from Brazilian rats and serological evidence of its widespread distribution in South America. *Am. J. Trop. Med. Hyg.* 34:810-15.

3. **Schmaljohn, C. S., S. E. Hasty, J. M. Dalrymple, J. W. LeDuc, H. W. Lee, C. H. von Bonsdorff, M. Brummer-Korvenkontio, A. Vaheri, T. F. Tsai, H. L. Regnery, D. Goldgaber, and P. W. Lee.** 1985. Antigenic and genetic properties place viruses linked to hemorrhagic fever with renal syndrome into a newly defined genus of Bunyaviridae. *Science* 227:1041-44.

4. **Schmaljohn, C. S., and J. M. Dalrymple.** 1983. Analysis of Hantaan virus RNA: evidence of a new genus of Bunyaviridae. *Virology* 131:482-91.

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FIGURE 1

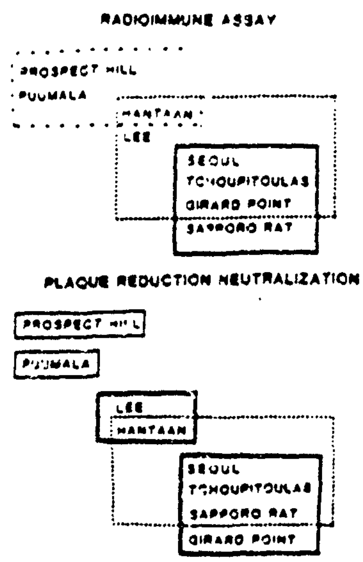

Figure 1. The serological relationships of eight hantaviruses determined by solid-phase radioimmune assay or plaque reduction neutralization are schematically compared. Virus isolates (and their rodent hosts) examined: three Korean isolates, prototype Hantaan virus (*Apodemus*), Seoul (*Rattus*), Lee (human); three U.S. isolates, Girard Point (*Rattus*), Tchoupitoulas (*Rattus*), and Prospect Hill (*Microtus*), one Japanese isolate, Sapporo rat (*Rattus*) and one Finnish isolate, Puumala (*Clethrionomys*).

FIGURE 2

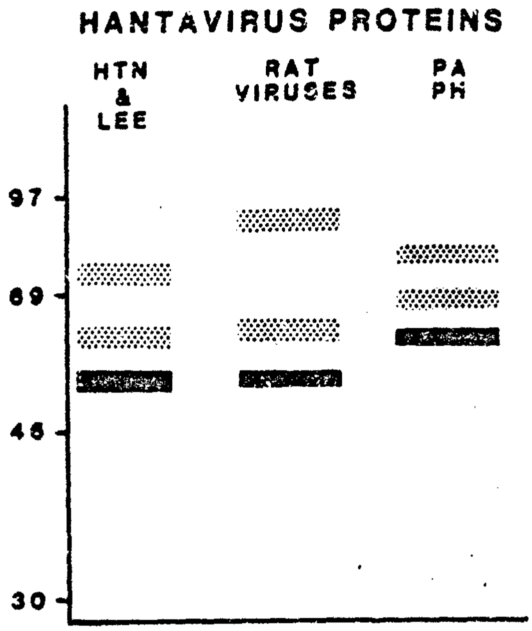

Figure 2. Polyacrylamide gel electrophoretic comparison of three distinct structural protein profiles detected are schematically illustrated. Groups included: (1) Hantaan (HTN) and Lee; (2) Rat viruses (Seoul, Girard Point, Tchoupitoulas, Sapporo Rat); (3) Puumala (PA) and Prospect Hill (PH).

FIGURE 3

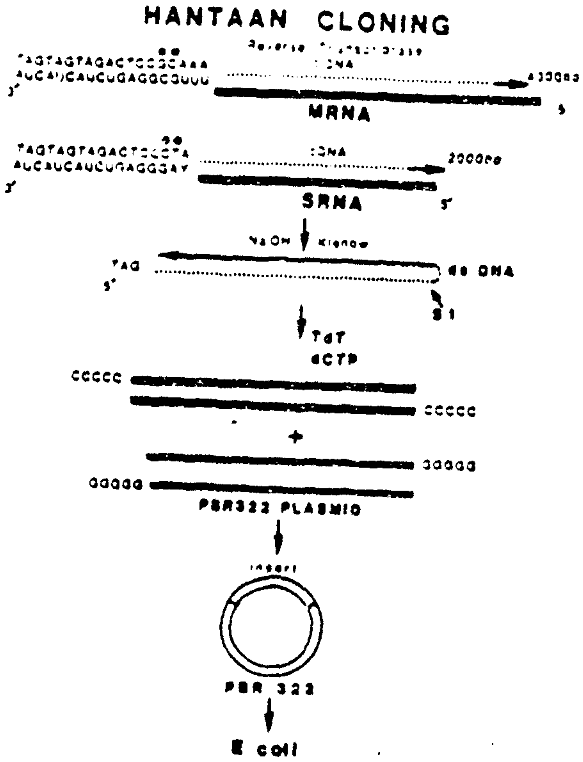

Figure 3. A diagram of the standard recombinant DNA procedures utilized for cloning the M and S genome segments of Hantaan virus. The sequences of the synthetic oligonucleotides used for first strand cDNA synthesis priming are displayed and the differences between the M and S oligonucleotide primers are indicated by *.

FIGURE 4

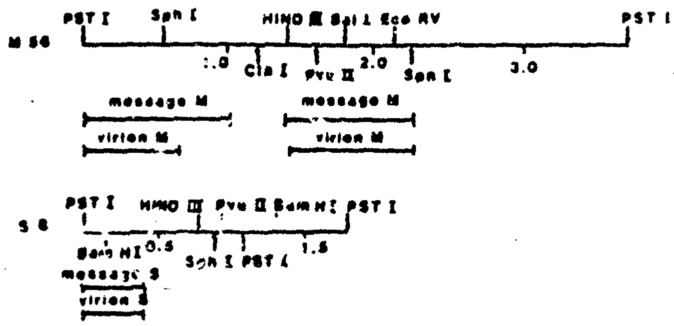

Figure 4. Partial restriction maps of the largest M (M56) and S (S8) cDNA clones obtained are diagrammed. The positions of the virion sense and message sense single stranded cDNA probes produced by subcloning into the M13 bacteriophage are depicted beneath each map.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DAOG 1536	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 84 10 01	4. KIND OF SUMMARY H. TERM	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AM	141
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) (U) Risk Assessment and Evaluation of Virus Agents and Their Vectors Under Laboratory Conditions

12. SUBJECT AREAS
0605 Clinical Medicine; 1503 Defense; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	4.5	742
c. TYPE	d. AMOUNT	86	0.0	0*
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Disease Assessment Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Bailey, C L
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-2775
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Faran, M E
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Arthropod Transmission; (U) Entomology; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Identify the intrinsic factors that influence the ability of arthropods to transmit viruses which may be actual or potential BW agents. Information will allow for development of methods for predicting relative risk of military personnel to arbovirus of BW importance and for designing specific vector control strategies.

24. (U) Ecologic and genetic factors relating to an arthropod's vector competence to transmit viruses are studied under controlled environments.

25. (U) 8410 - 8509 An avidin-biotin-peroxidase immunocytochemical procedure was developed and is being used to locate Rift Valley Fever (RVF) virus antigen in infected mosquito cells and in serial sections of infected *Culex pipiens* mosquitoes. This procedure will facilitate the study of intrinsic factors that affect the vector competence of mosquitoes exposed to RVF virus. Studies are nearing completion which demonstrate that the survival and reproduction potential of the mosquito *Cx. pipens* is adversly affected when they are infected with RVF virus. An electron microscopic study has been initiated to study the morphogenesis and pathogenesis of RVF virus in the salivary glands of *Cx. pipiens*.

*This research will be part of Work Unit 871-AB in FY86.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AM-141: Risk Assessment and Evaluation of Virus Agents and Their Vectors Under Laboratory Conditions

PRINCIPAL INVESTIGATOR: Charles L. Bailey, LTC, Ph.D.

ASSOCIATE INVESTIGATOR: Michael E. Faran, CPT, Ph.D.

Background:

Arthropod-borne diseases are transmitted by individual insects belonging to a particular species. It is the proportion of these individuals that determines the "vector competence" of a species in a geographical area. Vector competence of a species, therefore, is a populational phenomenon dependent on the overall susceptibility of the vector for the etiological agent and its subsequent ability to transmit that agent. In order to assess and predict which geographical areas constitute potential arthropod-borne-disease problems, it is necessary to estimate that proportion of the insect population which is vector-competent. Unfortunately, for the great majority of insect-borne diseases, this has never been possible because of the many variables associated with the agent-factor-man cycle. This research unit addresses several of these parameters regarding virus-mosquito vector interactions, specifically those associated with Rift Valley fever (RVF) virus susceptibility and transmission. For a more complete review of the intrinsic factors affecting vector competence and transovarial transmission, respectively, see Hardy et al. (1) and Burgdorfer and Tesh (2).

Summary:

The avidin-biotin-peroxidase (ABC) immunocytochemical procedure has been developed and is being used to locate RVF virus antigen in infected *Aedes albopictus*, C6/36 cultured cells and in serial sections of infected *Culex pipiens* mosquitoes. This technique opens new avenues of research by permitting the study of viral antigen distribution in mosquitoes at a level of detail not possible with enzyme-linked immunosorbent assays, fluorescent antibody techniques, or viral titration. The ABC procedure will thus facilitate the study of intrinsic factors that affect the vector competence of *Cx. pipiens* and other mosquitoes exposed to RVF virus, specifically, factors influencing the dissemination, morphogenesis, and pathogenesis of the virus. Currently, this methodology is being extended to the ultrastructural level with thin sections of mosquito tissue.

Completed studies on the effect of RVF virus infection on survival of adult *Cx. pipiens*. Although survival rates varied among trials, overall, *per os* exposure, and IT inoculation of virus resulted in an increase in mortality rate. Several statistical models are being used to test for fitness of the data.

A larval growth inhibitor or toxin was discovered in the mosquito, *Ae. palpalis*, which was collected in the Central African Republic this year.

We have begun a time-series, electron microscopic study of infection, morphogenesis, and pathogenesis of RVF virus in the salivary glands of *Cx. pipiens*.

Progress

The avidin-biotin-peroxidase complex, immunocytochemical procedure was evaluated for detection of RVF virus antigen in mosquito cells and in paraffin sections of mosquitoes.

The objective of this research was to adapt the avidin-biotin-peroxidase complex (ABC) staining technique to study the dissemination of RVF virus antigen in infected mosquito cells and serial paraffin sections of fixed whole mosquitoes. The highly specific, sensitive ABC technique (4,5) facilitates detection of antigen in tissue at a level of detail not possible by other methods. The high sensitivity of the ABC technique (6,7) is based on the strong affinity between the basic glycoprotein avidin (molecular weight approximately 68,000) and the low-molecular-weight vitamin, biotin. Since avidin has four biotin-binding sites, avidin may be labeled with various cytochemical marker molecules, such as biotinylated enzymes (horseradish peroxidase (4-6,8) or fluorochromes) (8), and still bind with one or more molecules of biotin. These labeled avidin molecules have vacant biotin-binding sites which can bind to biotinylated antibodies that have previously been bound to viral antigens. The appropriate epifluorescence or visible light microscope can then be used to visualize antigen-bound, avidin-biotin marker complexes.

Regardless of fixative, differential staining occurred in both the *Ae. albopictus* cells and in the *Cx. pipiens* sections. Infected *Ae. albopictus* cells consistly exhibited RFV virus antigen-specific staining with the mixture of mouse anti-RVF virus, monoclonal antibodies at dilutions of 1:500 and 1:1000, and for the polyclonal rabbit anti-RVF virus antibody at 1:200 and 1:400. There was specific staining when the mixture was used at dilutions as high as 1:10,000, but the proportion of positive cells decreased drastically. Among the different trials of primary antibodies within the optimum range, the proportion of infected cells positive for antigen varied from 10% to 95%. Within each trial, the proportion was relatively constant. The uninfected negative controls, tested at the above antibody dilutions, consistently had less than 1% of their cells nonspecifically stained. Antigen-specific stain was found only in the cytoplasm and not in the nuclei of the cells.

The highest dilution of the antibody mixture that produced specific staining in the *Cx. pipiens* was 1:5000 in one infected mosquito and 1:1000 in two others. All three uninfected specimens were negative.

Omission of the primary antibody, secondary antibody, ABC reagent, or diaminobenzidine from the procedure applied to sections resulted in the loss of specific staining. Likewise, sections of uninfected mosquitoes inoculated intrathoracially with only diluent did not stain. Sections which were inadvertently allowed to dry stained nonspecifically.

Preliminary Comments on specific tissues

Regions of melanized cuticle appear similar in color to positively stained tissues. A list of tissues showing specific staining is shown in Table 1. Outstanding among the specifically staining tissues was the fat body (Table 1). In all regions of the body, this tissue was antigen positive. The epidermis was infected either throughout the body or it had preferentially concentrated viral antigen from the hemolymph. Although the epidermis was only a single cell thick, it may be significant in viral amplification, because it covers the entire organism and is involved in all other structures of ectodermal origen. All regions of the alimentary canal demonstrated specific staining. Staining in the fore- and hindgut is indicative of a disseminated infection. However, a negative does not necessarily indicate a nondisseminated infection, because a certain amount of time is required after the virus emerges from the midgut until other tissues become infected. The Malpighian tubules were the only organs of the alimentary canal that showed non-specific staining. For the circulatory system, the heart, aorta, and specifically hemocytes stained for viral antigen; therefore, it appeared that the hemocytes, as well as the plasma, could be involved in the dissemination of the virus. Only the pericardial cells and nephrocytes stained non-specifically. Skeletal muscle did not exhibit any specific staining, whereas the visceral muscle associated with the alimentary canal did stain, indicating the presence of viral antigen. With the exception of the neuropile and fibers, all other structures of the central, peripheral, and stomatogastric nervous system stained specifically. Of particular interest was the staining of the major endocrine tissues. It is possible that this phenomenon may be the result of infection of the stomatogastric nervous system. Specific staining occurred in all genital tissue except for the oocytes, nurse cells, and follicular epithelium. These data support the observation of the inability of this species to transovarially transmit RVF virus. The salivary glands exhibited specific staining in all regions.

The ABC technique is a sensitive method for locating RVF viral antigen in fixed cultured C6/36 *Ae. albopictus* cells and in sections of *Cx. pipiens*. The staining was highly specific at practical working dilutions of the primary antibody, and there was very little background reaction. In the *Ae. albopictus* cells and in the *Cx. pipiens* tissue, staining was limited to the cytoplasm.

The nonspecific staining in the Malpighian tubule cells possibly was due to diaminobenzidine reacting with urate spheres, or perhaps to antibodies or the ABC complexes adhering non-specifically. The cause of the nonspecific reactivity to the DAB of the pericardial cells was not apparent. Note that the color of melanin in many cuticular structures (e.g., trachael intima, pharyngeal pump) was similar to the color of the polymerized diaminobenzidine, but these structures were easy to identify and did not present a problem.

Currently, we are using the ABC technique to follow the dissemination of RFV viral antigen from the posterior midgut epithelium to other tissues of the mosquito with respect to time. Also we are attempting to adapt this technique to electron microscopy in order to investigate viral morphogenesis and pathogenesis in the cells of different organs. This approach should prove useful in elucidating the nature of the different "barriers" to dissemination of virus in the midgut, ovaries, and salivary gland.

PRESENTATIONS:

1. **Faran, M. E., R. F. Tammariello, R. G. Routier, and C. L. Bailey.** Spatial and temporal dissemination of Rift Valley fever virus in orally infected *Culex pipiens*. Presented, Annual meeting of American Society of Tropical Medicine and Hygiene, 3-6 December, 1984.

2. **Tammariello, R. F., M. E. Faran, J. Meegan, and C. L. Bailey.** Application of ELISA for detection of Rift Valley fever virus antigen in mosquitoes. Presented, Annual meeting of American Society of Tropical Medicine and Hygiene, 3-6 December, 1984.

3. **Romoser, W. S., M. E. Faran, R. G. Routier, and C. L. Bailey.** Rift Valley fever antigen detection in mosquito tissues by the avidin-biotin-peroxidase complex technique. Presented, Annual meeting of American Mosquito Control Association, 19 March, 1985.

4. **Faran, M. E.** Infection, dissemination and transmission of Rift Valley fever virus by *Culex pipiens*. Guest lecturer at University of Maryland, Department of Entomology, 13 May, 1985.

PUBLICATIONS:

1. **Faran, M. E., C. Burnett, J. J. Crockett, and W. L. Lawson.** 1984. A computerized mosquito information and collection management system for systematic research and medical entomology (Diptera: Culicidae). *Mosq. Syst.* **16**:289-307.

2. **Faran, M. E., W. S. Romoser, R. G. Routier, and C. L. Bailey.** 1985. Use of the avidin-biotin-peroxidase complex immunocytochemical procedure for detection of Rift Valley fever virus antigen in paraffin sections of mosquitoes. *Submitted to Am. J. Trop. Med. Hyg.*

Table 1. Rift Valley Fever virus antigen as determined in tissue of avidin-biotin-peroxidase complex procedure. The mosquito is *Culex pipiens* infected by *per os* or intrathoracic (IT) inoculation.

Tissue	*Pc. Os** - Disseminated		IT* - INFECTED		UNINFECTED*
Fat body	90	(9/10)	100	(25/25)	0 (0/4)
Epidermis	75	(6/ 8)	100	(23/23)	0 (0/4)
Alimentary canal					
Esophagus	87.5	(7/ 8)	100	(21/21)	0 (0/4)
Diverticula	88.9	(8/ 9)	100	(23/23)	0 (0/4)
Anterior midgut	100	(11/11)	17.4	(4/23)	0 (0/4)
Posterior midgut	100	(10/10)	16.6	(4/24)	0 (0/4)
Malpighian tubules	100	(9/ 9)	83.3	(20/24)	100 (4/4)
Hindgut	87.5	(7/ 8)	55.6	(10/18)	0 (0/4)
Gut muscle	100	(8/ 8)	100	(13/13)	0 (0/4)
Circulatory system					
Heart	28.6	(2/ 7)	60	(12/20)	0 (0/4)
Aorta	16.7	(1/ 6)	44.4	(8/ 8)	0 (0/3)
Pericardial cells	71.4	(5/ 7)	94.1	(16/17)	100 (4/4)
Nephrocytes	100	(1/ 1)	100	(2/ 2)	100 (4/4)
Hemocytes	100	(3/ 3)	100	(6/ 6)	0 (0/4)
Skeletal Muscle	0	(0/ 8)	0	(0/23)	0 (0/4)
Nervous System (Central + Peripheral)					
Cell bodies	88.9	(8/ 9)	92	(23/25)	0 (0/4)
Neuropile + fibers	0	(0/ 7)	0	(0/24)	0 (0/4)
Ommatidia	80	(8/10)	100	(25/25)	0 (0/4)
Johnston's organ	71.4	(5/ 7)	53.9	(7/13)	0 (0/4)
Nervous system (stomatogastric)					
Corpus cardiacum	50	(2/ 4)	33.3	(3/ 9)	0 (0/4)
Corpora allata	83.3	(5/ 6)	87.5	(14/16)	0 (0/4)
Reproductive System					
Oocytes	0	(0/ 6)	0	(0/22)	0 (0/4)
Oviducts and Ovarian sheath	33.3	(2/ 6)	10	(2/20)	0 (0/4)
Salivary glands	88.9	(8/ 9)	100	(22/22)	0 (0/4)

*Percentage equals $\frac{\text{number positive}}{\text{number examined}} \times 100$

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA307136	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 85 05 20	4. KIND OF SUMMARY K. TERM	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AP	142
b. CONTRIBUTING				
c. CONTRIBUTING	DA LRRDAP, F786- 01			

11. TITLE (Precede with Security Classification Code)
(U) Artificial Intelligence and Expert Systems in BW Defense

12. SUBJECT AREAS
0902 Computers; 1201 Mathematics and Statistics; 0904 Information Theory

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
85 04	85 09	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	0.3	45
c. TYPE	d. AMOUNT	86	0.0	0
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Administrative Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Higbee, G A
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7514
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Meadors, G F
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Robotics; (U) Artificial Intelligence; (U) Expert Systems; (U) BW Defense; (U) RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop applications of artificial intelligence and expert systems (AI/ES) in the DA and DoD medical defense program.

24. (U) Initially, short- and long-range AI/ES application areas will be identified and coordinated with other DoD agencies. Contracts will be established to address specific DA and DoD BW defense requirements.

25. (U) 8505 - 8509 - During the four months since the establishment of this work unit, efforts have been threefold: (a) identification of possible applications of AI technology in the BW defense program, (b) acquisition of application software that uses AI techniques, and (c) plans for the development of appropriate AI software via contract and in-house efforts.

This work unit has been spread out into most of the other in-house work units and constitutes an integral part of each.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYM
	DA 302643	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	H. TERM	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AN	143
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Rickettsial Diseases of Potential Biological Warfare Importance

12. SUBJECT AREAS 0613 Microbiology; 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	85 10	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	1.9	69
c. TYPE	d. AMOUNT	86	0.0	0
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Airborne Diseases Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Stephenson, H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Sanchez, V
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Vodkin, M H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Q Fever (U) Coxiella burnetii; (U) Lab Animals; (U) Mice; (U) Rickettsial Diseases

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Study well-recognized and newly discovered rickettsiae with acknowledged BW potential, which require special containment. Assess and characterize virulence factors, growth requirements, pathogenesis in animal models, and resultant therapeutic implications that will provide a base for developing and evaluating vaccines to protect US military personnel.

24. (U) Determine the molecular aspects of virulence and immunogenesis, develop animal models, and characterize biochemical, pathologic, and immunologic alterations accompanying infection. Apply advanced methodology to define immunogenic components of the pathogens. Information is used to produce efficacious prophylactic and therapeutic regimens.

25. (U) 8410-8509-Peritoneal macrophages of mice resistant, moderately susceptible, and sensitive to *Coxiella burnetii* infection were evaluated for activation. Inactivated phase I whole cells of *C. burnetii*, but not infection, activated the peritoneal macrophages of all three strains of mice, as determined by both nonspecific tumoricidal and reduction in 5'-AMPase activities

Continued under DA302643.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AN-143: Rickettsial Diseases of Potential Biological Warfare Importance

PRINCIPAL INVESTIGATOR: E. H. Stephenson, D.V.M., Ph.D., COL, VC

ASSOCIATE INVESTIGATORS: V. Sanchez, Ph.D.
M. H. Vodkin, Ph.D.

Background:

Among the rickettsiae, *Coxiella burnetii* has been recognized as a potential BW agent. Investigations have continued, therefore, to define the pathogenesis and immunogenesis of airborne-induced infection. The role of the macrophage in the pathogenetic scheme was examined by using inbred mouse strains of various degrees of susceptibility to *C. burnetii*.

Summary:

Peritoneal macrophages from mice resistant, moderately susceptible, and sensitive to *C. burnetii* infection were evaluated for activation. Inactivated, phase I, whole cells of *C. burnetii*, but not infection, activated the peritoneal macrophages of all three strains of mice, as determined by both non-specific tumoricidal and reduction in 5'-AMPase activities.

Progress:

Activation of peritoneal macrophages was assessed in strains of mice resistant (C3H/HeN), moderately susceptible (BALB/cJ), and sensitive (A/J) to phase I *C. burnetii* infection. Peritoneal macrophages were elicited by a single i.p. injection of either inactivated phase I whole cells or viable phase I *C. burnetii*. Tumoricidal and 5'-AMPase assays were performed on days 0, 1, 3, 5, 7, and 10 after injection. With inactivated whole cells, significant ($P < 0.05$) tumoricidal activity was observed as early as days 1 and 3 with C3H/HeN and BALB/cJ, respectively, and on day 10 with A/J. Peritoneal macrophages from mice infected with *C. burnetii* were tumoricidal on day 7 for C3H/HeN and on 7 and 10 for A/J, whereas the peritoneal macrophages of BALB/cJ were not tumoricidal. Injection of inactivated whole cells effected significant reduction of surface 5'-AMPase activity of peritoneal macrophages from all three strains of mice. Infection of C3H/HeN and A/J did not reduce 5'-AMPase activity; however, the BALB/cJ strain showed significant reductions. Thus, *C burnetii* inactivated whole cells, but not infection, activated peritoneal macrophages of all three strains of mice for both nonspecific turmoricidal and reduction in 5'-AMPase activities.

PRESENTATIONS:

1. Sanchez, V., J. C. Williams, and E. H. Stephenson. Activation of peritoneal macrophages of various strains of mice by *Coxiella burnetii*. Presented, American Society of Rickettsiology, Laguna Beach, CA, Feb 85.

2. **Sanchez, V., J. C. Williams, and E. H. Stephenson.** Activation of peritoneal macrophages of various strains of mice by *Coxiella burnetii.* Presented, 85th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, Mar 85.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 3815	85 10 10	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 10	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AH	146
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-5.2/3			

11. TITLE *(Precede with Security Classification Code)* Exploratory Research for Antiviral Drugs

12. SUBJECT AREAS 1503 Defense; 0605 Clinical Medicine; 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		85	3.5	432
c. TYPE	d. AMOUNT	86	3.0	546
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Canonico, P G
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-2290
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Ussery, M A
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Kende, M

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U)Antiviral Drugs; (U)Pharmacology; (U)Viral Diseases; (U)Lab Animals;(U) Mice;(U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Conduct laboratory studies to develop novel antiviral drugs by identifying potential targets for pharmacologic intervention. These drugs are needed to treat soldiers who may become sick with viral infections.

24. (U) Describe mechanisms of action and metabolism of new drugs and provide analytical support for drug analysis. Perform structure-activity analyses to identify new analogs for synthesis. Apply approaches of molecular virology and cell biology to define the molecular basis of virus binding, uptake, uncoating, replication, and maturation.

25. (U) 504 compounds were tested for activity against a number of RNA viruses, but none was sufficiently effective to merit further development. Ribavirin, a known antiviral, was found to be effective against Hantaan virus, the causative agent of Korean hemorrhagic fever, in a suckling mouse model. Treatment with 50 mg/kg ribavirin begun on day 10, following onset of early clinical signs, saved 11 of 20 mice compared to 0 of 70 controls. Pharmacokinetic studies with human recombinant interferons in uninfected African green monkeys with interferon (IFN) alpha alone, IFN gamma, and both combined, showed that serum levels that may be effective could be achieved without measurable side effects, such as pyrexia, anorexia, or serum chemistry changes.

BODY OF REPORT

PROJECT NO: 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AH-146: Exploratory Research for Antiviral Drugs

PRINCIPAL INVESTIGATOR: Peter G. Canonico, Ph.D.

ASSOCIATE INVESTIGATORS: Meir Kende, Ph.D.
Michael A. Ussery, CPT, MSC
John H. Huggins, Ph.D.

Background:

Much of the antiviral research to date has focused on commercially exploitable drugs against herpes, influenza, and rhinoviruses. However, the viruses of the toga-, bunya-, and arenaviridae families, often considered to be "exotic" tropical viruses, are now beginning to receive special attention. This research program has focused on the discovery and development of agents or procedures which will be effective against a wide variety of "exotic" RNA viruses (1).

The program maintains an extensive antiviral drug screening effort. Initial testing consists of in vitro assays of a battery of RNA viruses and evaluation of rodent models. Other approaches being evaluated are the targeting of antiviral agents to specific tissue sites (2). This approach involves the synthesis of compounds which have inherent capacities to concentrate in specific organs. Another aspect of this approach is the coupling of antivirals to carriers molecules; this will promote the site-specific delivery of the drug.

Research activity is also directed at combination chemotherapy. Combinations of certain antivirals have been shown to exhibit significant synergy. We previously demonstrated synergistic antiviral effects against RNA viruses for combinations of ribavirin with either of two novel nucleotide derivatives, tiazofurin and selenazole. The synergy of drugs, especially ribavirin, with compounds which enhance the immune system is of special interest. Finally, the number of natural products which nonspecifically activate the immune system, resulting in enhanced host resistance to viral infections, has grown significantly due to the efforts of the National Institutes of Health (3). USAMRIID's program is evaluating such compounds as antiviral agents, as well as exploiting approaches for the specific targeted delivery of these immunomodulators, administered either alone or in combination with other antivirals.

Summary:

Five hundred compounds were tested for activity against a number of RNA viruses, but none was sufficiently effective to merit further development. Ribavirin, a known antiviral, was found to be effective against Hantaan virus, the causative agent of Korean hemorrhagic fever, in a suckling mouse model. Treatment with 50 mg/kg of ribavirin, begun on day 10, following onset of early clinical signs, saved 11 of 20 mice, compared to 0 of 70 controls. Picabanil, a new immunodulatory compound, was moderately efficacious against Rift Valley fever virus (RVFV) in mice. The compound elicited only low levels of interferon, but induced a strong cytotoxic activity of Natural Killer (NK) cells. An African green monkey

model was developed for yellow fever. Because of the moderate nature of the infection, the model offers a valuable tool to evaluate antiviral compounds and immunomodulators.

Progress:

Antiviral Screening Five hundred and four compounds, sixty of which were close analogs or derivatives of ribavirin, were screened for antiviral activity. Each compound was tested in vitro for efficacy against RVF, VEE, PIC, YF, SFS, VSV, JEV, and KHF. A list of compounds demonstrating activity against any of these viruses is given in Table 1. None of these compounds was more efficacious or less toxic then ribavirin and none showed activity against RVFV or VEE in mice.

Model development

A. A model was developed for detection of immunostimulatory compounds which restore an impaired immune response. The immune response of mice was abolished by physical means of immunosuppression, with irradiation of 600 Rads cobalt. Irradiation did not appear to increase the mean time-of-death of RVFV-challenged mice, and it considerably but not entirely reduced the antiviral efficacy of poly(ICLC) in a time-dependent fashion. When poly(ICLC) was given simultaneously with irradiation, there was a diminution of efficacy; however, the mean time-of-death remained prolonged significantly. Poly(ICLC) retained its complete immunostimulatory efficacy when administered 6 and 24 h before irradiation. At two or four days after irradiation the drug was fully and partially active, respectively. This model will be employed to measure the restoration of the anti-RVFV antibodies with poly(ICLC), and the cellular T-cell response with interleukin-2 (INT-2). The same principle will be used with chemically (cyclophosphamide)-induced immune suppression as an integrated part of compound characterization.

B. Characterization of yellow fever virus infection in the African green monkey-Barbados strain Yellow fever (YF) virus infection was charactorized in the African green monkey-Barbados substrain (AG-B). African green and rhesus monkey were described to be sensitive to human recombinant γ-interferon. Yellow fever infection in rhesus monkey is characterized by a rapid course of liver necrosis, a 100% lethality, making it difficult to evaluate antiviral compounds when given more than a few hours after infection. Therefore, we explored the sensitivity of AG-B monkey to YF in terms of lethality, viremia, changes in hematological parameters, and liver and kidney function enzymes.

Four monkeys were injected s.c. with 400 PFU (~10,000 mouse LD_{50}) of Dakah or Asibi strains of YF virus and monitored for clinical signs of disease. Viremia was detected in three out of four and four out of four Dakah or Asibi strain-infected monkeys, respectively, with viremia being about 30% higher in Asibi infected monkeys. One monkey which was injected with Dakah YF virus, died on day 23, although this monkey, as well as others, had no detectable virus titer on day 7 or later. Histopathological examination confirmed the presence of liver lesions characteristic of YF. SGPT and SGOT liver function enzymes were elevated in five of eight monkeys, with the highest values detected in the deceased monkey. There were no changes in liver-function enzymes in non-viremic monkeys. Glucose and urea nitrogen were elevated only in the monkey which died. CK and LDH enzymes were elevated in seven of eight monkeys, but the specificity of these enzymes have to be confirmed with isoenzyme analyses. Changes in enzyme levels were much more pronounced in those monkeys which were infected with the Asibi strain. The intensity of changes are comparable to those occurring in YF-infected rhesus monkeys. All surviving monkeys developed high titers of neutralizing antibodies. These observations make the AG-B monkey a very useful model for advanced testing of antivirals and immunomodulators. Furthermore, active compounds can be evaluated against mild, medium, or severe models of YF infection akin to human cases.

C. Japanese encephalitis virus in weanling mice Two strains of inbred weanling mice (BALB/cJ and C57Bl/6J) were infected i.p. with two strains of Japanese encephalitis virus (JEV) (Peking and 2372-Thailand). The i.p. infection of BALB/cJ mice with 10^6 PFU of JEV 2372 resulted in a mortality rate of 70%. Clinical signs of infection included anorexia, weight loss, partial paralysis of limbs, full paralysis, behavioral changes and, in some cases, diarrhea. These signs were basically consistent with observations in human and monkey JEV. The approximate LD_{50} is 1.8 X 10^4 PFU. The average time of death was 7.7 days for 10^6 PFU/mouse, 14.7 days for 10^3 PFU/mouse, and 19 days for 1 PFU/mouse.

BALB/cJ mice infected with the Peking strain of JEV showed an approximate LD_{50} of 17 PFU. The average time of death was 8.9 days for 10^6 PFU/mouse, 10.6 days for 10^3 PFU/mouse, and 10 days for 1 PFU/mouse.

C57Bl/6J mice infected with JEV 2372 showed an approximate LD_{50} of 3 X 10^3 PFU. The average time of death was day 9 for 10^6 PFU/mouse, day 12 for 10^3 PFU/mouse, and day 13 for 1 PFU/mouse.

C57Bl/6J mice infected i.p. with the Peking strain of JEV showed an approximate LD_{50} of 20 PFU. The average day of death was day 9 for 10^6 PFU/mouse, day 11 for 10^3 PFU/mouse, and day 12 for 1 PFU/mouse.

Brain, heart, lung, kidney, liver, and spleen were removed upon necropsy and homogenized for virus isolation. Almost all brains yielded high-titer JEV. Infectious virus could not be isolated from the other five organs examined. Thus, inbred weanling mice provide an excellent model for JEV and will be used in subsequent studies of candidate antiviral compounds.

D. Intracranial infection of African green monkeys with JEV Two African green monkeys were infected i.c. with JEV 2372, a strain isolated from a fatal human case in 1979 in Thailand. Monkey T628 received 1 x 10^4 PFU in 0.1 ml i.c. and died on day 9 postinfection. JEV was isolated from the CSF on days 3 and 5 postinfection. Monkey T629 received 1 x 10^2 PFU in 0.1 ml i.c. and died on day 8 postinfection. Virus was isolated from the CSF on day 5 postinfection.

Thus JEV replicated and produced clinical disease very similar to that in the rhesus/cynomolgous model. Time-to-death may be slightly shorter than in rhesus monkeys (day 10-14), but too few animals have been studied to measure the significance of this difference. The African green monkey JEV model will be used in interferon (alpha and gamma) combination efficacy studies.

E. Development of therapy for hemorrhagic fevers The development of therapy for hemorrhagic fevers caused by arenaviruses has made significant progress with the discovery of the efficacy of ribavirin therapy for Lassa fever. Similar progress has not been made for hemorrhagic fever with renal syndrome (HFRS) or other hemorrhagic fevers, in part, due to the lack of an adequate animal model in which to evaluate therapeutic approaches.

Previous work has shown that Hantaan virus (HV), the prototype member of a group of related viruses etiologically associated with HFRS, inhibits ribavirin. In vitro studies have shown that Hantaan is among the most sensitive of RNA viruses to ribavirin (4).

The development of a suckling mouse model to study the pathogenesis of Hantaan (5) served as a starting place for evaluating ribavirin against this important class of viruses, although the requirement of a suckling mouse model (the only symptomatic model developed to-date) posed considerable problems due to age-dependent toxicity.

Ribavirin therapy was evaluated by using suckling mice infected at 24-h of age. In this model, mice were viremic by day 6 and exhibited both virus and viral antigen in tissues by day 8. Weight loss occurred, beginning on day 10. Starting on day 11, there was a short period of hyperexcitability, followed by onset of clinical signs of disease: ruffled fur, hunched posture, and diminished mobility on the next day. Fluorescent antibody was detected on day 14, followed by neutralizing antibody ou day 15, with a concurrent disappearance of viremia. Animals developed paralysis of both hind limbs and began to die by day 16. All infected animals died with a mean time-to death of 21 days (2).

In previous ribavirin studies, treatment was initiated at three different stages postinfection, with doses ranging from 0 to 100 mg/kg, continued over 14 days. Infected groups consisted of no treatment; treatment begun on day 6 at the onset of viremia; on day 10 at the onset of weight loss, when virus and viral antigen are present in all major tissue; and, on day 14 at the first appearance of antibody and the onset of severe clinical signs. Unrelated animals infected with 10 LD_{50} of HV (strain 76/118) showed weight loss beginning on day 10 and developed paralysis of both hind limbs on days 15 to 18, followed by death between days 20 and 21.

Treatment initiated on day 10, at a period when virus was present at high concentrations in all organs and early clinical signs were evident, increased both survival and mean time-to-death. Ribavirin at 25 mg/kg increased survival to 30% ($P < 0.05$), while 50 mg/kg increased survival to 55% ($P < 0.001$) and mean time-to-death to greater than 75 days. Ribavirin-treated animals that died did so at a later time than placebo-treated animals. Appearance of clinical signs was also reduced in a dose-dependent manner; all signs disappeared by day 30 in survivors, with no sign of disease recurrence over a 75-day follow-up period. Untreated and placebo-treated mice showed a similar weight loss followed by death. In contrast, animals treated at 25 and 50 mg/kg did not show weight loss, and began weight gain normally following the cessation of drug treatment on day 24.

To characterize the mechanism of protection, a serial sacrifice study was undertaken employing the most promising treatment regimens: 25 mg/kg began on day 6, and 60 mg/kg began on day 10, with corresponding controls of placebo treatment and no treatment. Experimental design was similar to that of the preceeding studies. Organ titers for treatment begun on day 10 with 50 mg/kg were compared to sham treatment. Virus titers were near peak when treatment was initiated. Following initiation of treatment on day 10, serum virus titers fell significantly within 24 h. Virus decreased within two days in liver and spleen, in lung within six days, and kidney by eight days. Treated survivors continued to show decreasing virus titers through day 24, when treatment was stopped. By day 18, virus titers in organs of treated animals were two logs lower than sham-treated controls, with the exception of the brain. Virus titers in brain fell by day 20, when virus in untreated animals reached > 10^7 PFU/g; death occurred between days 20 and 21. The fluorescent antibody response, although delayed by two days in ribavirin-treated animals, followed a course similar to that in placebo-treated or untreated animals. Neutralizing antibody appeared at the same time as in controls. Virus titers in animals first treated on day six were similar, although virus reduction was less dramatic.

An age-dependent toxicity of ribavirin placed a restriction on the utility of the model, but such toxicity noted in newborn mice was not unexpected. Successful therapy of Hantaan virus-infected suckling mice with ribavirin could be initiated as late as day 10, following the onset of early clinical signs and the appearance of virus in serum and organs. This survival is especially impressive, because drug toxicity peculiar to suckling mice limits the maximum testable dose to dosages that are suboptimal for cures in other Bunyaviridae mouse models. Survival of Hantaan virus-infected mice cannot be linked specifically to reduction in circulating and visceral organ viral titers. A temporal relacionship exists, however, and viral titers are clearly linked to survival in many disease systems. In HFRS of humans, pathological changes are most consistent with vascular changes and tissue damage caused by immune-mediated mechanisms. Immune complexes and complement activities have been demonstrated in Far Eastern and Scandinavian variants of HFRS. Immune mechanisms involving the humoral or cellular systems have been suggested for Hantaan pathogenesis. The humoral antibody response was delayed, relative to sham-treated animals, with a normal response in both indirect fluorescent and neutralizing antibody. Thus, no indication of humoral immunosuppression by the drug was seen.

Antiviral Activity of Immunomodulators

Preliminary studies with Picabanil, a polysaccharide extract of Group A streptococci, indicate a moderate antiviral activity against RVFV. Administration of two or three doses of 5 mg/kg of Picabanil with treatment beginning one to three days prior to challenge increased about 3-fold the median survival time of mice and resulted in 30% long-term survivors. Administration of a single dose of 5 mg/kg Picabanil to mice one to four days prior to sacrifice elicited a high degree of spleenic NK cell, cytotoxic activity against tumor target cells. The augmentation of NK cell activity was equal to that obtained with administration of poly(ICLC) and *P. acnes*. Augmentation of liver NK cell cytotoxicity following a single dose of 5 mg/kg persisted for 18 days, although the best results were obtained between one and four days postinjection. Due to its long-lasting effects, this compound may be valuable in synergistic studies.

Avridin, a lipoidal amine, showed moderate efficacy against RVFV when administered with intralipid or in liposome-encapsulated form. Studies to improve and confirm the antiviral activity of this compound have yet to be performed.

The antiviral activity of human recombinant INT-2 was assayed in mice against RVFV. The compound was completely inactive when given for 10 days on either prophylactic and/or therapeutic schedules in doses ranging from 1000 to 100,000 U/mice. Since, INT-2 has a short, 7- to 10- min half-life, it was tested by using a minipump for continuous delivery. The minipump did not improve the activity of INT-2, however the rate of drug delivery was not determined.

Combination efficacy studies with ribavirin and immunomodulators Previous efficacy studies with poly(ICLC) revealed that doses lower than 20 μg were therapeutically less effective or not effective at all, unless frequently administered. Therefore, to improve the practical application of Poly(ICLC), a combination chemotherapy approach was tried using 1 µg poly(ICLC), and 12.5, 25 and 50 mg/kg ribavirin. Therapy was started 24 h postchallenge with 250 PFU of RVFV. Efficacy was evaluated by ranking regimens according to the incremental, relative risk-of-death against a standard treatment with 1 µg poly (ICLC) as described by the Cox model. Treatment with three regimens of ribavirin was not more effective than the standard treatment, the relative risk-of-death being two times higher or lower than with 12.5 and 50 mg/kg ribavirin, respectively. However, the incremental relative risk of mortality with 1 µg poly (ICLC) in combination with 12.5, 25, and 50 mg/kg ribavirin was reduced 5-, 10-, and 30-fold, respectively, indicating a significant therapeutic synergism.

Additive therapeutic effects were obtained for combinations of ribavirin and liposome-encapsulated, muramyl tripeptide derivative or ribavirin and Picabanil. Efficacy was increased only about twofold in comparison with treatments of single compounds. More extensive studies are planned to exploit the full potential of these combinations against RVFV and other viral infections.

Therapeutic application of liposome-mediated drug targeting of ribavirin against RVFV infection in mice were continued. In the past, we have employed only multilamellar vesicles (MLV), because of their ease of preparation and relative stability on storage. Variable results were obtained when MLV were used one- and two-days postchallenge. It was proposed that a variable rate of uptake of MLV with a heterologous size distribution might be responsible for their results. Relatively large liposomes, with either one or a few lamallae (SUV), appear to be more suitable for drug delivery to the reticuloendothelial cells. Although the yield of drug encapsulation was identical to MLV and SUV, the latter appeared to be more efficacious in two out of three therapeutic studies. However, neither carrier was consistently more effective than the aqueous drug.

Prophylactic efficacy of triacetyl ribavirin (RTA) against JEV in peripherally infected mice C57Bl/6J mice were injected i.p. twice a day with drug from day -1 to day +7 postinfection with JEV 2372 (10^5 PFU/mouse). Daily doses were 228, 152, 76, and 0 mg/kg RTA, or the equivalent doses of 150, 100, 50, and 0 mg/kg ribavirin. Mortality rates were higher in both ribavirin and RTA than in untreated, uninfected mice. There was little evidence that RTA significantly increased time-to-death or any other parameter of infection. Brain titers of JEV upon necropsy were higher with increasing doses of either ribavirin or RTA. Thus RTA was not effective against JEV in mice. Further studies are designed to measure i.c. drug concentrations to better evaluate RTA and other prodrug analogues of ribavirin for ability to cross the blood-brain barrier.

Ability of RTA to cross the blood-brain barrier. A method of perfusing mouse brain to remove contaminating, drug-containing, capillary blood has been developed. Perfused brains are than homogenized, deproteinized, and analyzed by HPLC, allowing a comparison of serum and central nervous system drug concentrations. This technique allows the capability of a drug to cross the blood-brain barrier to be determined.

Intrathecal administration of ribavirin and other antivirals Ribavirin was administered intrathecally (i.t.) to monkeys at the following dosages daily for 5 days: 0.05 mg/kg, 0.5 mg/kg, 2.0 mg/kg, 5.0 mg/kg, and 25 mg/kg. All monkeys experienced a mild, reversible anemia that can be attributed solely to the number of blood samples taken, since the same anemia was present in the control monkey treated i.t. with Hank's Balanced Salt Solution (HBSS). The monkeys treated with 0.05 and 0.5 mg/kg showed no ill effects of treatment. Monkeys treated with 2.0 and 5.0 mg/kg showed a reversible depression of appetite on days 2, 3, 4, and 5 of treatment. One dose of 25 mg/kg ribavirin proved fatal. Serial serum and CSF samples are being processed by HPLC to determine i.t. treatment pharmacokinetics. Two monkeys received 3 mg/kg i.t. RTA under the same treatment schedule with no contraindications.

PRESENTATIONS:

1. **Kende, M.** Therapy of Rift Valley fever virus infection with poly(ICLC) and ribavirin. Presented, Interscience Conference on Antimicrobial Agents and Chemotherapy, 1984.

2. **Kende, M.** Liposomes as carriers of ribavirin and immunomodulators for treatment of Rift Valley fever. Presented, Gordon Research Conference, Plymouth, NH., 1984.

3. **Kende, M.** Treatment modalities of murine Rift Valley fever virus infection. Presented, Czechoslovak (CS) Society of Microbiology of the CS Academy of Sciences, Bechyne, CS, 1985.

4. **Canonico, P. G., M. Kende, B. J. Luscri, and J. W. Huggins.** 1985. Chemotherapy of toga and bunyaviruses with liposome-mediated targeting and combination of antiviral agents. Abstract of the 6th International Congress of Virology.

5. **Canonico, P. G., M. Kende, B. J. Luscri, and J. W. Huggins.** 1985. Chemotherapy of toga and bunyaviruses with liposome-mediated targeting and combination of antiviral agents. Abstracts of the Inter-American Society for Chemotherapy. In Press.

6. **Ussery, M. A., P. G. Canonico, G. S. Ward, M. R. Elwell, and D. S. Burke.** 1985. Ribavirin triacetate in the treatment of murine and primate Japanese encephalitis. Presented at the Annual Meeting of the American Society for Virology, Albuquerque, New Mexico.

PUBLICATIONS:

1. **Kende, M., C. R. Alving, W. L. Rill, G. M. Swartz, Jr., and P. G. Canonico.** 1985. Enchanced efficacy of liposome-encapsulated ribavirin against Rift Valley fever virus infections in mice. *Antimicrob. Agents Chemother.* 27:903-907.

2. **Kende, M.** 1985. Prophylactic and therapeutic efficacy of poly(ICLC) against Rift Valley fever virus infection in mice. *J. Biol. Resp. Modif.* In Press.

3. **Wood, S. G., K. G. Upadhya, N. K. Dalley, P. A. McKernan, P. G. Canonico, R. K. Robins, and G. R. Revankar.** 1985. Synthesis and Biological activity of 5-Thiobredinin and certain related 5-substituted imidazole-4-carboxamide ribonucleosides. *J. Med. Chem.* 28:1198-1203.

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2. **Gangemi, J. R.** 1985. Selective targeting of antiviral and immunomodulatory agents in the treatment of arenavirus infection, pp. 1-44. Annual Report, DAMD 17-84-C-4144.

3. **Levy, H. B., and F. L. Riley.** 1982. A comparison of immune modulating effects of interferon and interferon inducers, pp. 303-322. *In* M. Landy, (ed.). *Lymphokines*. Academic Press, New York.

4. **Kirsi, J. J., J. A. North, P. A. McKernan, B. K. Murray, P. G. Canonico, J. W. Huggins, P. C. Srivastava, and R. K. Robins.** 1983. Broad-spectrum antiviral activity of 2-β-D-ribofuranoselenazole-4-carboxamide, a new antiviral agent. *Antimicrob. Agents Chemother.* **24**:353-361.

5. **Kim, R. G., and K. T. McKee.** 1985. Pathogenesis of Hantaan virus infection in suckling mice: clinical, virologic, and serologic observations. *Am. J. Trop. Med. Hyg.* **34**:388-395.

Table 1. Therapeutic index* of new antiviral compounds

Contractor I.D.	AVS I.D.	Therapeutic Index (MTC/ID50)							
		RVF	VEE	PIC	YF	SF	VSV	JE	KHF
SN-141	1177	-	-	-	.425	-	-	-	*
SN-138	1180	-	-	-	1.398	-	-	-	*
SR-61	1183	12.73	-	-	-	*	-	*	*
BJ64052	1190	-	4.39	-	-	*	-	*	*
BJ64070	1191	-	-	-	-	*	.261	*	*
BJ84983	1192	25.000	-	-	-	*	-	*	*
EH96013	1195	4.123	-	-	-	*	-	*	*
A5-520-9	1200	-	-	2.38	-	*	.861	*	*
A6428	1444	4.82	-	-	-	9.31	-	-	*
B3044	1445	-	-	-	-	8.31	-	-	*
B4147	1446	-	-	-	-	2.17	-	1.87	*
B4148	1447	7.90	-	17.71	-	12.19	-	15.80	*
E2904	1452	1.92	-	4.38	-	-	-	1.68	*
F2308	1453	4.18	1.79	2.55	-	2.27	1.66	-	*
F3336	1454	3.34	-	2.77	-	-	-	3.33	*
U6331	1490	-	7.99	6.30	-	6.62	6.87	6.77	*
U643	1493	-	-	-	-	.367	-	.394	*
Y3499	1503	-	-	8.52	-	-	-	-	*
A657U	1566	*	*	*	*	*	*	24.5	*
A9992U	1589	*	*	*	*	*	*	5.98	*

*Therapeutic Index: a measure of the antiviral potential of the drug calculated as MTC/ID_{50}.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DAOG 3810	85 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AB	150
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code) Exploratory Research for Protection Against Botulism

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense; 0620 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE / EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE / d. AMOUNT
e. KIND OF AWARD / f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	2.6	536
86	0.0	0*

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Crumrine, M H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7211
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Siegel, L S
MILITARY/CIVILIAN APPLICATION: M	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Microbial-Toxins; (U) Vaccines; (U) Toxoids; (U) Ram I; (U) Lab Animals; (U) Mice

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop and evaluate biologics and selected compounds for prevention and treatment of diseases induced by microbial toxins of military importance. Prepare, characterize and produce toxiods/vaccines suitable for protection against botulism and other toxins. Collect, produce and test immunoglobulins having efficacy in prevention and treatment of microbial toxemias. Develop materials and methods for detection and assay of toxins.

24. (U) Develop new technology for fermentor-system production of small experimental lots of microbial toxins and methodology for isolation, purification, alteration and detection of toxins. Immunogenicity of various antigens will be described and converted to experimental toxiods/vaccines. Improve procedures for immuno- and chemotherapy of toxin-mediated diseases. Action(s) and effects of toxins and their antagonists will be determined and described at the cellular level.

25. (U) 8410-8509-The effect of 3,4-diaminopyridine (3,4-DAP) on the survival of mice poisoned with types A, B, E, or F botulinal toxin was evaluated. Treatment with 3,4-DAP significantly prolonged the survival of mice poisoned with type A toxin but was not effective in the treatment of mice poisoned with type B, E, or F toxin. 3,4-DAP prolonged survival but did not alter the final outcome of poisoning with these toxins. Once treatment was stopped, mice died as a consequence of the intoxication.

*This research will be part of Work Unit 871AF in FY 86.

BODY OF REPORT

PROJECT NO. 3M162770A871: Medical Defense Against Biological Warfare

WORK UNIT NO. 871-AB-150: Exploratory Research for Protection Against Botulism

PRINCIPAL INVESTIGATOR: Martin H. Crumrine, MAJ, Ph.D.

ASSOCIATE INVESTIGATORS: L. S. Siegel, Ph.D.
A. D. Johnson-Winegar, Ph.D.
L. C. Sellin, Ph.D.

Background:

Since World War II, microbial toxins have been recognized as agents with possible military significance. Of the many microbial toxins identified as having military importance based upon their lethality, stability, numerous routes of delivery, simplicity of production, and availability, botulinal toxins emerged as likely candidates for use as overt, covert, or terrorist biological agents. There are seven known serotypes (neurotoxins A-G) and man is highly susceptible to minute amounts (nanogram quanities) of these toxins.

Protection against these toxins can be achieved by several mechanisms of activ and passive immunization. Man can be immunized with chemically inactivated toxins (toxoids); and, hence, toxin effects can be prevented. Current toxoids do not contain types F or G and thus do not afford complete protection against all serotypes of botulinal neurotoxins. Antitoxin produced in horses against types A-E has been utilized in the therapy of patients with botulism. These products lack activity against types F and G neurotoxins and, due to their heterologous protein nature, produce an unacceptable number of adverse reactions. Although the therapeutic efficacy of antitoxin is questionable, sufficient amounts do offer limited treatment benefit; thus, production of equine antitoxins with activity against all types of botulinal neurotoxins are needed. Also, mechanisms to eliminate the adverse reactions to equine serum must be developed. A candidate drug (3,4-diaminopyridine) is being evaluated as a possible antagonist to the paralysis produced by these neurotoxins.

Summary:

The effect of 3,4-diaminopyridine (3,4-DAP) on the survival of mice poisoned with types A, B, E, or F botulinal toxin was evaluated. Treatment with 3,4-DAP significantly prolonged the survival of mice poisoned with type A toxin but was not effective in the treatment of mice poisoned with type B, E, or F toxin. 3,4-DAP prolonged survival but did not alter the final outcome of poisoning with these toxins. Once treatment was stopped, mice died as a consequence of the intoxication.

Progress:

To determine the efficacy of 3,4-diaminopyridine (3,4-DAP) as a potential treatment for botulism, its effect on the survival times of mice injected with type A, B, E, or F botulinum toxin was examined. At zero time, a group of mice (30 or more) were injected i.p. with 10, 20, or 40 LD_{50} of botulinum toxin. Three hours later, when the mice showed signs characteristic of botulism (ruffling of the fur and labored abdominal breathing), half of each group of mice was treated with 3,4-DAP. The drug (phosphate salt) was administered by i.p. injection at a dose of 4 or 8 mg/kg, beginning at 3 h and at hourly intervals thereafter. Mice were examined at 15-min intervals for survival. The pattern of survival of untreated versus treated groups was compared by using a computer statistical program, Survival Analysis. A P value ≤ 0.05 was considered statistically significant. Results are summarized in Table I.

At each dose of type A toxin tested, treatment with 3,4-DAP prolonged the survival of the mice. When the patterns of survival of untreated versus treated groups of mice were compared, statistically significant differences were apparent at all doses of type A toxin tested. (Groups of mice receiving 3,4-DAP only were not affected by the injections of the drug.) In contrast, treatment with 3,4-DAP did not significantly increase the survival time of mice injected with type B, E, or F toxin (Table 1).

For 20 to 40 LD_{50} of type E toxin, statistical analysis of the survival pattern of treated versus untreated mice for each experiment did not demonstrate significant differences. In one experiment with 10 LD_{50}, treatment with 3,4-DAP shortened survival time ($P < 0.50$). Increasing the dosage of the drug to 8 mg/kg did not increase the survival time of mice receiving 10, 20, or 40 LD_{50} of type E toxin. With type B toxin, there was a statistically significant difference ($P < 0.05$) in the pattern of survival in three experiments. In two of these, treatment with 3,4-DAP prolonged survival, but in the third, the drug shortened the time to death. However, the differences in the median survival time of treated versus untreated mice were, at most, 1 h, and treatment did not consistently prolong survival. Administration of 3,4-DAP did not prolong the life of mice receiving 10, 20, or 40 LD_{50} of type F toxin. In two experiments with 20 LD_{50} of type F toxin, use of 3,4-DAP actually decreased survival time. Thus, 3,4-DAP prolonged survival of mice that had received type A botulinum toxin, but was not effective against type B, E, or F toxin at the concentrations of drug and toxin tested.

These observations support data from previous studies that suggest antigenically distinct types of botulinum toxin differ in pharmacological activity. Results of these investigations demonstrate that, in this case, extrapolating information obtained with one serotype of botulinum toxin to other serotypes is unwarranted, and emphasizes the need for comparative pharmacological studies with these neurotoxins.

Synthetic peptide vaccines Dr. Siegel is evaluating synthetic peptides, produced according to known sequences of the neurotoxin molecule, for their efficacy as vaccines (a collaborative effort with Dr. J. Schmidt). Peptides, each synthesized from a known amino acid sequence unique to type A, B, or E neurotoxin, have been coupled to carrier molecules and used to immunize rabbits. Sera from these animals are being evaluated for antibody effective against the native toxin.

In a collaborative effort, Drs. Schmidt and Siegel have devised a new purification scheme for type E neurotoxin that uses an HPLC system. This new procedure has reduced the number of steps required to obtain a pure product. Furthermore, due to the use of state-of-the-art instrumentation, this purification method is very rapid (minutes rather than days) and produces high yields of neurotoxin, with excellent recovery of biological activity.

Studies to evaluate methods for the purification of type F neurotoxin using classical protein purification techniques are continuing. Thus far, the procedure yields a preparation that is approximately 80% pure. (Neurotoxin comprises 0.1% or less of the protein in the culture fluid.)

Studies to evaluate a solid phase capture system (nitrocellulose membrane) for enzyme immunoassay of botulinal toxins were completed. Initial studies involved methods development to confirm sensitivity claims of the manufacturer. Known concentrations of human IgG were attached to the nitrocellulose solid phase according to the manufacturers protocol. The unbound sites were then blocked with bovine serum albumin and rabbit anti-human IgG antibody conjugated to horseradish peroxidase was added to the wells containing the human IgG. The membrane was washed and an insoluble chromogenic substrate was added to each well to visualize the binding of the conjugate with human IgG. Parallel assays were run utilizing standard polycarbonate 96-well ELISA plates and an identical protocol to detect human IgG. Sensitivity of the reference system for human IgG was consistantly ≤ 1 ng/ml. The nitrocellulose-based system was continuously plagued by nonspecific background and other mechanical problems that prevented the system from reaching the sensitivity of the standard polycarbonate plate system. This system was considered to be too unreliable and insensitive for further consideration as a laboratory detection assay and can not be recommended for further evaluation as a basis for rapid detection of antigens in a field setting.

<u>Summary of Dr. B. R. DasGupta's Contract Work</u> A lumbrical muscle preparation for the study of botulinal neurotoxins has been developed. Dose-response curves for types A and E (intact and trypsinized) toxin have been established and will be used as reference points for further studies of toxin binding. Experiments were conducted to determine if the binding of isolated heavy chains of type A botulinal toxin could alter the binding or paralytic effects of intact toxin. Preincubation of heavy chains with neuromuscular junctions significantly delayed the effects of intact toxin. Apparently, the heavy chain binds to the same sites as the intact toxin. Purified heavy chains from type B toxin formed pH- and voltage-dependent channels in planar bilar membranes. These findings are similar to those observed for the heavy chain of diphtheria and tetanus toxins. The light chains from type B toxin had no effect under the same experimental conditions. These findings give further support to the conclusion that the heavy chain carries the binding portion of these toxin molecules. Other structure-function studies have confirmed that modification of the histidine moities in type E toxin results in complete loss of toxicity. This nontoxic molecule can then be used as an immunizing agent to induce neutralizing antibodies. Recent confirmatory studies have repeated the original findings and may give us additional options in creating toxoids in the future.

PRESENTATIONS:

1. **Siegel, L. S.** Effect of 3,4-diaminopyridine on the survival of mice receiving botulinum toxins. Presented at the USAMRIID Professional Staff Conference. 1985.

2. **Crumrine, M. H.** Development of an ELISA for botulinum toxins. Presented at USAMRIID Professional Staff Conference. 1984.

PUBLICATIONS:

1. **Kauffman, J. A., J. F. Way, Jr., L. S. Siegel, and L. C. Sellin.** 1985. Comparison of the action of types A and F botulinum toxin at the rat neuromuscular junction. *Toxicol. Appl. Pharmacol.* 79:211-217.

2. **Siegel, L. S., A. D. Johnson-Winegar, and L. C. Sellin.** 1985. Effect of 3,4-diaminopyridine on the survival of mice injected with botulinum neurotoxin type A, B, E or F. *Toxicol. Appl. Pharmacol.* (Submitted).

Table 1 Effect of 3,4-Diaminopyridine Treatment on Survival of Mice Injected Intraperitoneally with Botulinum Toxin

Botulinum Toxin		Survival Time (h)				
		Untreated[a]		3,4-Diaminopyridine[a]		
Type	LD_{50}	Mean ± SE[b]	Median	Mean ± SE	Median	P
A[c]	40	5.7 ± 0.23	5.2	14.5 ± 0.47	>15.0	<0.001
		8.5 ± 0.53	8.6	14.7 ± 0.95	>16.0	<0.001
		6.2 ± 0.58	5.8	10.8 ± 0.98	10.5	<0.002
	20	9.1 ± 0.78	8.3	15.0 ± 0	>15.0	<0.001
		10.2 ± 0.87	9.2	15.1 ± 0.85	>16.0	<0.001
		7.7 ± 0.67	6.8	10.7 ± 0.95	12.1	<0.05
	10	12.3 ± 0.62	11.9	14.8 ± 0.22	>15.0	<0.001
		13.1 ± 0.72	13.9	14.3 ± 1.1	>16.0	<0.05
B[c]	40	6.1 ± 0.46	6.0	6.9 ± 0.62	6.8	NS[e]
		6.6 ± 0.59	6.4	5.5 ± 0.50	5.6	<0.05 DF[f]
	20	7.0 ± 0.56	7.2	7.6 ± 0.38	8.3	<0.05
		7.8 ± 0.74	7.4	7.5 ± 0.46	7.6	NS
	10	9.7 ± 0.51	9.7	8.4 ± 0.51	8.7	NS
		8.8 ± 0.48	9.1	10.4 ± 0.57	10.2	<0.05
E[c]	40	5.8 ± 0.54	5.8	4.5 ± 0.34	5.2	NS
	20	6.9 ± 0.76	6.3	5.9 ± 0.31	6.7	NS
		6.4 ± 0.28	7.2	7.0 ± 0.46	7.2	NS
	10	7.5 ± 0.51	8.1	8.2 ± 0.63	8.3	NS
		9.3 ± 0.28	9.6	7.5 ± 0.56	7.5	<0.05 DF
F[c]	40	4.6 ± 0.35	4.9	4.6 ± 0.18	5.2	NS
		5.7 ± 0.48	5.7	5.6 ± 0.54	5.3	NS
		5.1 ± 0.34	5.4	6.1 ± 0.61	5.6	NS
	20	7.0 ± 0.45	7.2	5.6 ± 0.40	5.8	<0.05 DF
		7.1 ± 0.36	7.4	5.7 ± 0.28	6.1	<0.01 DF
		6.7 ± 0.32	7.2	6.1 ± 0.30	6.5	NS
	10	7.7 ± 0.38	8.3	7.1 ± 0.26	7.3	NS
E[d]	40	4.3 ± 0.34	4.7	4.4 ± 0.34	4.8	NS
	20	5.3 ± 0.45	6.0	5.7 ± 0.30	6.0	NS
		5.5 ± 0.49	5.5	5.7 ± 0.62	5.8	NS
	10	7.8 ± 0.36	8.5	7.2 ± 0.40	8.1	NS
		6.4 ± 0.55	6.6	6.6 ± 0.46	7.4	NS

[a] Number of mice in each of the treated and untreated groups
[b] Mean survival time (Kaplan-Meier) ± standard error of the mean
[c] 3,4-DAP (phosphate salt) at 4 mg/kg
[d] 3,4-DAP (phosphate salt) at 8 mg/kg
[e] Not significant (P>0.05)
[f] 3,4-DAP treatment shortened survival time

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 307135	2. DATE OF SUMMARY 85 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 85 05 20	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	131
b. CONTRIBUTING				
c. CONTRIBUTING	NONE			

11. TITLE (Precede with Security Classification Code) (U) Isolation and Characterization of Immunogenic Components of Anthrax Toxin

12. SUBJECT AREAS 0613 Microbiology; 1503 Defense; 0601 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
85 04	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	0.5	98
c. TYPE	d. AMOUNT	86	1.0	110
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Bacteriology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Welkos, S L
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7341
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available)
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) BW Defense; (U) Lab Animals; (U) Mice; (U) Guinea Pigs; (U) Rabbits; (U) Anthrax Toxin; (U) Lac Fusion Proteins; (U) Cloning

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To isolate polypeptide fragments of anthrax toxin components by using plasmid cloning vectors that allow construction of hybrid genes. To characterize the structure, immunogenicity, and biological activity of the hybrid toxin proteins. This work may ultimately lead to an improved anthrax vaccine to protect military personnel.

24. (U) Isolate protective antigen (PA)-lac Z gene fusions and characterize these fusions by using restriction endonuclease digestion and agarose gel electrophoresis. Once purified, the different PA-lac hybrids will be assayed by in vitro and in vivo techniques. Purified, fully characterized hybrid proteins might be conjugated with other anthrax antigenic material to form a new vaccine.

25. The PA gene of *B. anthracis* was inserted into the new vector. Numerous mutants were isolated which have different lengths of the inserted DNA fused to the detector gene (encoding β-gal). Six to seven mutant classes were identified based on the size of the deletion in the cloned DNA. The PA-containing fusion proteins are being purified using a new column chromatography procedure. The proteins are being characterized according to their size (by migration on gel), their immunological activity (by binding to anti-PA antibodies), and their biological activity. PA fusion proteins found to be immunologically active but nontoxic will be used to vaccinate animals. We developed an inbred mouse model for studying anthrax which should be suitable for vaccination/challenge studies.

BODY OF REPORT

PROJECT NO. 3A161101A91C: In House Laboratory Independent Research

WORK UNIT NO. 91C-00-131: Isolation and Characterization of Immunogenic Components of Anthrax Toxin

PRINCIPAL INVESTIGATOR: Susan L. Welkos, Ph.D.

Background:

Bacillus anthracis produces a three-part exotoxin which appears to have an essential role in the disease manifestations of anthrax (1, 2). This toxin is composed of the protein components protective antigen (PA), edema factor (EF), and lethal factor (LF). A major goal of research in anthrax is to clone the genes encoding the individual components into vectors that will: (a) simplify analysis of the structure, biological activity, and immunogenic potential of the toxins; and (b) allow sufficient quantities of toxin protein to be purified for vaccine studies. This research plan addresses these goals, with emphasis on the first one.

The initial recombinant work on anthrax toxin focused on cloning of the gene for PA. A 6-kilobase (Kb) fragment of DNA from *B. anthracis* plasmid pX01 was cloned into *E. coli* plasmid vector pBR322. Production of biologically active PA was shown (3). Additional work is ongoing to determine the sequence (approximately 2.5 Kb) within the cloned DNA that encodes the PA protein; identify the structural domains of PA associated with biologic and immunogenic activity; and improve the yield of PA by subcloning the gene into expression vectors. Recently *E. coli* fusion vectors have been developed to simplify cloning and subsequent analysis of gene products. These new plasmid vectors allow the construction of hybrid genes, composed of cloned DNA fused with the *lac* Z gene of *E. coli*. A hybrid protein consisting of the product of the cloned DNA fused to β-galactosidase, the product of the *lac* Z gene, is produced. It is easily detected based on the lactose-hydrolyzing activity of β-galactosidase (4-7). One fusion system which was recently developed appears to be especially useful for our studies with the PA molecule. The plasmid pMLB1034 carries a *lac* Z gene which is inactive due to the substitution of restriction enzyme cloning sites for the gene's initiation sequences. An entire gene of interest, with stop and start signals, is inserted into one of the cloning sites. This recombinant is then screened for the emergence of derivative clones having spontaneously occurring deletions in the plasmid DNA. The deletions of interest are those which remove the stop codon of the insert and fuse the inserted gene sequences in frame to *lac* Z. This simple step generates clones that are now positive for β-galactosidase, which is expressed by the hybrid protein product of the fused genes. By using this technique, it is possible to generate a series of gene fusions, each having differing lengths of the cloned gene fused to *lac* Z (4, 6). Analyses of the protein fusions that result would help to define the domains of the PA molecule responsible for induction of protective immunity, recognition of target cell receptors, and binding to LF and EF.

Bacillus anthracis is considered a potential biological warfare threat due to the stability of its spores and the nonspecific symptoms that accompany infection. Major goals of research in anthrax are to understand the pathogenesis of the infection and to develop an improved anthrax vaccine for administration to military personnel. These studies will contribute in the efforts to isolate and characterize the genes encoding anthrax toxin. Cloning of the toxin genes will facilitate the purification of each toxin component for vaccine studies and will aid research on the role of the components in disease.

Summary:

The PA gene of *B. anthracis* was inserted in the new vector. Numerous mutants were isolaed which had different lengths of the inserted DNA fused to the detector gene (encoding β-gal). Six to seven mutant classes were identified according to size of the deletion in the cloned DNA. The PA-containing fusion proteins are being purified by a new column chromatography procedure. Proteins are being characterized according to their size (by migration on gel), their immunological activity (by binding to anti-PA antibodies), and their biological activities. PA fusion proteins that are found to be immunologically active, but nontoxic will be used to vaccinate animals. We developed an inbred-mouse model for studying anthrax. This model should be suitable for vaccination/challenge studies.

Progress:

Our strategy for isolating PA-*lac* Z gene fusions was as follows: The fragment of DNA containing the gene for PA and originating from plasmid pSE36 was inserted next to the inactive *lac* Z gene of plasmid pMLB1034. The presence of the PA gene was confirmed by DNA hybridization, and production of intact PA was confirmed by ELISA and Western blot analyses. This recombinant was then used as the "parent" strain for the isolation of β-galactosidase-producing, gene-fusion derivatives. Many similar clones have been isolated, each consisting of a plasmid with varying lengths of PA-encoding DNA fused to the *lac* Z gene of the vector.

Several procedures will be used to characterize the PA gene fusions that have been presumptively isolated. First, size and location of the deletion in the fused gene will be determined. Restriction endonuclease digestion and agarose gel electrophoresis techniques will be used. Production of β-galactosidase by the fusions will be verified by an enzyme assay described by Miller (8); and the PA-*Lac* hybrid proteins will be visualized by polyacrylamide gel electrophoresis of cell lysates. The information from these experiments will allow us to correlate the size of the fused genes with the molecular weight of the hybrid proteins. In order to further explore the antigenicity and biological activity of the fusion proteins, we will attempt to purify and concentrate them. One promising procedure involves affinity chromatography was recently described for purification of *lac* hybrids (9, 10). Once purified, the different PA-*Lac* hybrids can be assayed for reactivity with anti-PA antibody and for biological activity. In vitro assays will determine the presence of domains on the PA fusions responsible for binding to eucaryotic cell receptors and/or to EF and LF. The Chinese hamster ovary cell assay for PA + EF activity, and a macrophage cytotoxicity test for PA + LF, are available for competitive binding assays with the PA-*Lac* proteins. (11, A. Friedlander - unpublished data). In vivo function of the hybrid proteins can be ascertained by the rat lethality bioassay for PA plus LF (12). In addition to characterizing the structure and biological activity of the PA-Lac fusions, we will immunize animals with the proteins to determine the antibody response to these altered PA molecules. Immunized animals will then be challenged with live virulent *B. anthracis* or with anthrax toxin to compare the protective efficacy of the different fusion proteins. Data obtained from these assays will be correlated with the structure of the PA-Lac fusion proteins, in order to define the specific immunodominant and biologically active domains of the PA molecule. Following isolation and characterization, the PA-*Lac* hybrid proteins will be potentially useful in several areas of the anthrax research program. (a) For example, hybrid proteins which vary in the size of the PA component could be used as unique probes for analyzing the specificity of the monoclonal, anti-PA antibodies being generated. The anti-PA antibodies would be screened for differential binding to the fusion proteins to determine the epitope-specificity of the antibodies. (b) Hybrid proteins could be used to confirm the DNA sequence that is being determined for the cloned PA gene. Correlation of the molecular weight of each fusion protein with the size of the deletion in the corresponding DNA will confirm the origin and direction of transcription of the PA gene on the cloned DNA fragment. (c) Finally, the fusions might be suitable components of a new vaccine for anthrax. PA-*Lac* proteins that are biologically inactive but immunologically reactive would be vaccine candidates. Hybrid proteins could be used unaltered or else manipulated enzymatically or genetically to separate the truncated PA polypeptides from the β-galactosidase component (13). Several multi-component vaccines are conceivable. The nontoxic PA fusion fragment could be combined with EF and/or LF to create a new chemical vaccine. Also, PA fusion proteins could be included with a candidate live vaccine strain to possibly enhance protection afforded by the latter. Klein et al. showed enhanced protection in guinea pigs when the animals were vaccinated with MDPH followed by Sterne organisms (14). Finally, a PA fusion protein could be conjugated to purified anthrax capsular material or cell-wall polysaccharide to create a vaccine similar to the polysaccharide-toxoid conjugates developed for *H. influenzae* and *N. meningitidis* (15, 16).

If gene fusion technology proves to be a successful approach to the characterization of the gene encoding PA, it could be a valuable tool for analyzing other genes of *B. anthracis*. These include the genes encoding EF and LF; the structural genes for other virulence factors and protective antigens, such as the poly-D-glutamic acid capsule; and the regulatory genes of *B. anthracis* that control synthesis of toxin, capsule, etc.

In summary, a hybrid gene fusion system was proposed as an approach to cloning and characterizing the toxin genes of *B. anthracis*. Hybrid PA-*Lac* genes are being isolated. If the PA fusion proteins are poorly expressed by the *E. coli* recombinants or cannot be purified adequately, other vectors designed to enhance expression of the cloned toxin gene will be investigated. Several plasmid vectors are available which contain strong promotors of *E. coli* or *B. subtilis* to direct transcription of the cloned gene. Examples of promotors used in high-level expression vectors include the lipoprotein gene and lactose operon promotors of *E. coli* (17).

PRESENTATIONS: None

PUBLICATIONS: None

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 301613	85 07 24	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	K.COMPLET		U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	136
b. CONTRIBUTING				
c. CONTRIBUTING	NONE			

11. TITLE (Precede with Security Classification Code) (U)Molecular Mechanism of Action of T-2 Mycotoxin

12. SUBJECT AREAS 1503 Defense; 0613 Microbiology; 0620 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 04	85 04		C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	1.0	113
c. TYPE	d. AMOUNT	85	1.5	200
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Middlebrook, J L
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7211
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available)
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) BW Defense; (U) T-2 Mycotoxin; (U) Trichothecene; (U) Yellow Rain; (U) Low Molecular Weight Toxin

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To study the molecular events leading to T-2 toxicity in cell cultures and to use the insights gained to develop and test possible therapeutic countermeasures for protection of U.S. military personnel.

24. (U) Using cell culture and subcellular systems, elucidate the events involved during the interaction of the trichothecene toxins with target cells. The mycotoxin T-2 will be used as a prototype. Radiolabeled T-2 is available and will be employed to follow the course of toxin binding to the cell, internalization, distribution, metabolism and elimination.

25. (U) 8310 - 8409 - Membrane potential is important in the T-2-cell association process since the ionophores monensin or nigericin both markedly increased the level of cellular T-2. Extrusion system responsible for cell cleansing behaves as an active transport system. Proved that the T-2 resistant cells we developed are deficient in the binding and/or transport of that and other tricothecenes. Quantified many aspects of T-2 binding to isolated ribosomes. In particular, measured the rapidity of attachment, rate of disassociation, affinity of binding, competition by related tricothecenes and some unrelated compounds. Virtually all tricothecenes bind to the same site on the ribosome as does T-2. Binding of T-2 to both the transport receptor and ribosome is stereospecific. Showed that the receptor or transport system clearly exists and is very important for two reasons: (a) most other trichothecenes bind to this same system so protection at this stage of action would be expected to cross-protect from a large number of trichothecene toxins; (b) since all trichothecene toxins created have approximately the same binding affinity to ribosomes yet differ in cell toxicity by a thousand or more fold, we conclude that transport system is the major factor determining a trichothecene toxin's potency.

BODY OF REPORT

PROJECT NO. 3A161101A91C: In-House Laboratory Independent Research

WORK UNIT NO. 91C-00-136: Molecular Mechanism of Action of T-2 Mycotoxin

PRINCIPAL INVESTIGATOR: J. L. Middlebrook, Ph.D.

Background:

Trichothecene toxins have been implicated as agents in the so-called "yellow rain" in Southeast Asia and Afganistan. These or similar agents could possibly be employed against U.S. or allied forces. At present, there are no established methods for the medical prevention or treatment of exposures.

The mechanisms by which trichothecenes cause death are not understood. The development of protective drugs in such a void is basically hit-and-miss. It is probable that an understanding of the molecular events involved when the toxin reaches its target would be helpful in designing possible therapeutic agents.

Summary:

We have shown that T-2 and T-2 tetraol bind to the same site on ribosomes and (within a factor of 1.5) equally tightly.

We have shown that T-2 tetraol does not bind to the same transport system on cells that T-2 does and that the tetraol-cell association is only about 1/100 of that seen with T-2.

We have found that the rate for tetraol extrusion by the cell is about the same as for T-2 so the reason for lower overall cell association must be a slower uptake of tetraol than T-2. We conclude the THE factor determing the inhibition of protein synthesis by T-2 and its metabolites is at the level of the cell membrane, presumably a receptor and/or transport system.

We found that T-2 cell association was markedly increased by energy deprivation, while T-2 tetraol cell association was not. Several possible ways of promoting cellular energy levels were studied to see if protection from T-2 was obtained. None of the treatments were helpful.

Progress

The kinetics of T-2-ribosome binding were studied. At 37°C, the association of T-2 with ribosomes was biphasic. In the initial phase, binding increased rapidly to what appeared to be a plateau equilibrium state by 30 min. However, if the incubation was continued further, a second phase was observed in which the binding gradually declined to essentially baseline values. In contrast, T-2-ribosome binding at 4°C was much slower, taking 24 to 48 h to reach completion. Continuing the incubation for up to an additional 24 h at 4°C did not result in the appearance of a descending limb of binding.

One likely explanation for the latter stage decline in T-2-ribosome binding at 37°C is thermal degradation of the ribosome. To test that possibility, we incubated ribosomes at 37°C and at various times, removed aliquots, and incubated them with ^{3}H-T-2 for 30 min at 37°C. Incubation at 37°C resulted in an immediate loss of binding capacity, a trend which continued to baseline values. The kinetics of this loss were roughly linear and did not describe a straight line when plotted semilogarithmically. For comparative purposes, we examined the rate of degradation of ribosomes prebound with T-2 at 4°C. In that case, there was a time lag of approximately 4 h before the apparent degradation process began. In addition, there was a small, but reproducible, increase in the binding immediately after the temperature was raised to 37°C. Despite the delay in its inception, the thermal degradation rates of T-2-bound or -unbound ribosomes were, within experimental error, the same as judged by the slopes for the two lines. Other experiments demonstrated that the T-2 binding capacity of ribosomes held at 4°C required 3 weeks to decay 50%.

The reversibility of T-2-ribosome binding was demonstrated by a "chase" experiment. After prebinding the ribosomes for 30 min with radiolabeled T-2 at 37°C, a 100-fold molar excess of unlabeled T-2 was added and incubation continued. The addition of a chase induced a prompt, substantial reduction in radiolabeled T-2-ribosome binding. Unlike the patterns seen in the degradation experiments, kinetics for chase-induced loss of binding were logarithmic. The apparent first-order rate constant for dissociation was 0.12 h^{-1} (1/2 time 2.5 h). Treatment of the data was not extended past 4 h since it appeared that spontaneous loss of binding became significant. Although dissociation of ribosomal-bound T-2 could be readily demonstrated at 37°C, the binding was apparently much tighter at 4°C. When chase experiments were carried out at that temperature, no measurable dissociation was observed for up to 24 h.

Binding isotherms for T-2-ribosome association at 4°C were performed. Because it was technically difficult to grow the large quantities of CHO cells required for ribosome preparation, we performed most binding experiments with yeast; cell-derived ribosomes. In almost every variable, T-2-yeast ribosome and T-2-CHO ribosome binding were indistinguishable. As the concentration of added T-2 increased, so did the level of binding. At all concentrations examined, the addition of a 100-fold excess of unlabeled T-2 competed for 90% or greater of the radiolabeled T-2-ribosome binding, indicating a specificity to the interaction. When analyzed by the method of Scatchard, the data described straight lines with high correlation coefficients. The slopes and y intercepts provided estimates of dissociation constants and sites per ribosome. For five separate experiments using yeast ribosomes, the values for each parameter were $2.93 \pm 0.28 \times 10^{-8} M^{-1}$ and 1.07 ± 0.02 sites/ribosome.

We made another approach to determine the dissociation constant for T-2-ribosome binding level for a constant amount of radiolabeled T-2 with increasing (competitive) amounts of unlabeled T-2. That concentration of unlabeled T-2 which blocked 50% of the binding should be an estimate of the dissociation constant. When performed, these experiments gave values of 6×10^{-8} M as the dissociation constant at both 4°C and 37°C. Although the binding assays were reproducible from the standpoint of replicate precision, there was some day-to-day variation in the position of a dose-response curve. In order to make a controlled comparison of the competition of T-2 for ^{3}H-T-2 binding to CHO cells versus CHO cell ribosomes, we performed the experiment at the same time using the same dilutions of competitor. The data presented a pattern in which competition curves with cells at 4°C and ribosomes at both 4°C and 37°C were identical, whereas the curve for cells at 37°C was always positioned to the left of the others.

An epimer of T-2 (termed β-T-2) was produced from the naturally occurring, stereochemically pure toxin (α-T-2) during the radiolabeling procedure. We found that β-T-2 was considerably less potent than α-T-2 at inhibiting cellular protein synthesis and also was bound to or taken up by cells at a much reduced level. We compared the binding of α- and β-T-2 with ribosomes and found that this interaction, too, was stereospecific. There was much more ribosomal binding by the α- as compared to the β-isomer. At most concentrations, the ratio of bound α- to β-T-2 was 20:1. In spite of the differences in levels of ribosomal binding by α- and β-T-2, both toxins appeared to bind at the same site. When the competition of unlabeled α-T-2 for ribosomal binding of radiolabeled α- or β-T-2 was compared, the two curves fell virtually on top of one another. Although it is possible that the inhibition of β-T-2-ribosome binding is allosteric, the similar shape and placement of the curves strongly suggest both toxins bind to the same ribosomal site.

Further competition studies were carried out with several other trichothecenes. Generally speaking, those toxins equal to or greater than T-2 at inhibiting protein synthesis competed very well for binding. Those less potent than T-2 were correspondingly less able to block its binding to ribosomes. For example, we found the macrocyclic trichothecene Verucarrin A was slightly more potent at inhibiting protein synthesis in CHO cells than was T-2. Verrucarrin A was as efficient as T-2 at competiting for ^{3}H-T-2-ribosomal binding. An example of the converse was deoxynivalinol. This trichothecene was $\leq$ 100-fold potent at inhibiting protein synthesis than T-2. Correspondingly, about 100-fold more verrucarol than T-2 was required to block 50% of the ^{3}H-T-2 ribosome binding.

Several other inhibitors of protein synthesis were tested as potential competitors of T-2-ribosomal binding; two were found to be of special interest. Anisomycin, an inhibitor of eukaryotic protein synthesis, is structurally unrelated to T-2 and has been reported to have a higher binding affinity for ribosomes at 0°C than 30°C. We found that anisomycin competed for the binding of T-2 to ribosomes and that, in agreement with the literature, the binding of anisomycin was tighter at a reduced temperature. Anisomycin also competed for the association of T-2 with CHO cells, but with a strikingly different pattern. First, the competition was more effective at 37°C than 4°C, exactly the opposite of the interaction with isolated ribosomes and also unlike the competition pattern of T-2 itself. Second, the concentration for 50% competition was much less (ca. 1/200) with cells than ribosomes at physiological temperature. These data suggest a site or sites on (or in) CHO cells other than ribosomes to which both T-2 and anisomycin bind.

Even stronger evidence for this concept was provided by competition experiments with emetine. Emetine is the principal alkaloid of ipecac and, like anisomycin, has no obvious structural similarities to sesquiterpenes such as T-2. We found that emetine competed for ^{3}H-T-2-CHO cell association, while no block of labeled T-2-ribosome binding was observed over the entire concentration range tested. Emetine blocked ^{3}H-T-2-cell association more effectively at 37°C than 4°C, although the two curves appeared to merge at higher emetine concentrations. Several other protein synthesis inhibitors examined, including cycloheximide, puromycin, oligomycin, and chloramphenicol, had no detectible effect of T-2-ribosome binding.

We determined the dose-response curves for inhibition of CHO cell protein synthesis by T-2 and T-2-tetraol. Clearly, T-2-tetraol was less potent than its parent toxin. As judged by the concentrations required to inhibit protein synthesis 50%, T-2 was 150-200-fold more potent than its metabolite. We also determined the dose-response curves for T-2 and T-2-tetraol inhibition of protein synthesis in CHO cells resistant to T-2. While it was readily apparent that the cells were resistant to T-2, a corresponding resistance to T-2-tetraol was not observed.

When the association of radiolabeled T-2 and T-2-tetraol with CHO cell was examined, very different patterns were seen. The association of T-2 with CHO cells was blocked by homologous unlabeled toxin, demonstrating a high degree of specificity to the binding. The addition of excess, unlabeled T-2-tetraol had little or no effect on ^{3}H-T-2 association with CHO cells.

The association of ^{3}H-T-2-tetraol with CHO cells was much lower than T-2. In marked contrast to the situation described immediately above, homologous unlabeled toxin competed poorly for ^{3}H-T-2-tetraol-CHO cell association as did excess, unlabeled T-2. The specific, cell-associated toxin was therefore calculated to be much lower, approximately 1/50 that seen with T-2.

The association of T-2 and T-2-tetraol in CHO and R2 cells was determined. As would be suspected from the fact that the R cells were not cross-resistant to T-2-tetraol, the resistant cells took up the same amount of T-2-tetraol as T-2. Of course, the absolute amount of association was much lower in the case of T-2-tetraol with both cell lines.

Since the total toxin associated with cells is determined by the rate of uptake and extrusion, these parameters were both measured in comparative fashion for T-2 and T-2-tetraol. The rate of association of T-2 with CHO cells (37°C) was much greater than that for T-2-tetraol. On the other hand, the rate of out-transport of both toxins was approximately the same, when normalized for the initial number of toxin molecules bound to the cells.

The association of T-2 and T-2-tetraol with isolated ribosomes was examined. The absolute level of ribosomal binding of T-2-tetraol was approximately one-half that of T-2. Unlike the situation with cells, homologous, unlabeled toxins competed well for the binding of either toxin, as did heterologous toxins. The specific binding was calculated to be greater than 90% in both cases.

Because the amount of ^{3}H-T-2-tetraol we had was limited, we were unable to carry out a binding isotherm and Scatchard analysis of tetraol association with ribosomes. As an alternative, we compared the competition of unlabeled T-2 and T-2-tetraol for ^{3}H-T-2 binding to ribosomes. Data indicate very similar dissocation constants for the binding of both toxins to CHO cell ribosomes, while the apparent affinity of T-2-tatraol for yeast ribosomes was 6-10-fold lower. A similar experiment with CHO cells resulted in a small, but non-dose-related competition of T-2-tetraol (maximum concentration examined 10μg/ml) for ^{3}H-T-2-CHO cell binding.

Relative distributions of T-2 and T-2-tetraol between cells and the medium were compared with a freely diffusible molecule, water. The data indicate that, relative to water, T-2 is concentrated by the cells while T-2-tetraol is excluded.

In a separate series of studies, we examined the effects of steroids, metabolic inhibitors, and ionophores on T-2-cell interactions. Our basic finding was the treatment of cells with certain compounds from these three classes of drugs led to a marked increase in specific T-2-cell association. The effect was due primarily to accelerated uptake of the toxin with a smaller, but measurable inhibition of out-transport. When we tested the effects of these compounds on T-2-tetraol-cell association, markedly different results were obtained. Treatment of CHO cells with fluoride, progesterone, or nigericin led to increases of 400-500% in total T-2-cell association. In contrast, much smaller effects were noted on T-2-tetraol-cell association, results consistent with the notion that T-2 tetraol does not interact appreciably with the putative T-2 transport system.

PRESENTATIONS: None

PUBLICATIONS: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 303476	85 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DIS'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
84 10 01	D. CHANGE	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	138
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Application of Recombinant DNA Technology to Develop New Generation of Q Vaccines

12. SUBJECT AREAS 0613 Microbiology; 1503 Defense; 0601 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 02	86 01	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	1.0	154
86	1.0	150

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Airborne Diseases Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Vodkin, M H
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7453
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Stephenson, E H
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Williams, J C

22. KEYWORDS (Precede EACH with Security Classification Code) (U) DNA Recombinant Technology; (U) Vaccine; (U) Q Fever; (U) *Coxiella burnetii*

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) *Coxiella burnetii* is perceived to be a prime candidate for biological warfare (BW). Current vaccines are reasonably effective against this rickettsial disease, but cause many side-effects. This is a pioneering study to determine the feasibility of using DNA recombinant technology to develop an entire new generation of safe and highly effective vaccines against Q fever. If successful, U.S. troops can be immunized without lost time due to sterile abscesses and sore arms.

24. (U) Standard techniques of gene cloning will be used to transform a host bacterial cell with *Coxiella burnetii* DNA. Screening for proteins in recombinant clones will be pursued by a number of techniques. Any clone producing an antigen that is recognized by specific antisera becomes a candidate for a vaccine seed. This study was approved by Institutional Biosafety Committee of WRAIR representing DoD and the Office of Recombinant DNA Activities, National Institutes of Health (NIH).

25. (U) 8410-8509 - DNA restriction, fragment-length polymorphism was shown among a number of *Coxiella burnetii* isolates, and provides a marker for strain identification. A deletion of DNA arose in the transition of the Nine Mile strain from phase I to phase II. A clone was obtained that included the deleted segment of 19 kilobases. Restriction fragment profiles of most isolates are analogous. Plasmids of isolates from human endocarditis cases differ from the typical. Both types of plasmids were cloned as single pieces for each. The EcoRI fragment of the cardiotropic plasmid has little or no homology to any fragment of the other plasmid prototype.

BODY OF REPORT

PROJECT NO. 3A161101A91C: In-House Laboratory Independent Research

WORK UNIT NO. 91C-00-138: Application of Recombinant DNA Technology to Develop New Generation of Q Vaccines

PRINCIPAL INVESTIGATOR: M. H. Vodkin, Ph.D.

ASSOCIATE INVESTIGATORS: E. H. Stephenson, D.V.M., Ph.D., COL, VC
J. C. Williams, Ph.D., CDR, USPHS

Background:

Q fever, the disease caused by *Coxiella burnetii*, has a world-wide incidence. In humans, it most frequently leads to an acute, debilitating, pulmonary disease. In about 5% of these cases, a chronic form of the disease involving the liver (granuloma) or cardiac tissue (endocarditis) can ensue (1). The acute disease could be disruptive to military operations and the chronic disease would have long-term implications for both military and civilian populations in the absence of immunization. However, the currently available vaccine can produce undesirable reactions in a proportion of the vaccinees (2). There is evidence that reactogenic and immunogenic properties reside in different components of the organism (3). The main purpose of this work unit is to clone the DNA of the pathogen and screen the clones for production of immunogenic compounds. This approach will complement biochemical attempts to separate immunogens from reactogens and has the advantage of not requiring large amounts of biological material.

The prototype strain for *C. burnetii* is Nine Mile I. There is also an avirulent spontaneous derivative, Nine Mile II, which has undergone an apparently irreversible phase shift. A number of other isolates from various geographic locales, hosts, and vectors are available. Except for phase variation (I and II), there is no method for determining whether the isolates should be considered as taxonomic entities and whether strain variation will be a factor for evaluation of a vaccine. Another goal, therefore, would be some method for reliable strain identification.

Summary:

Chromosomal and plasmid DNAs have been extracted and analyzed from a number of isolates of *C. burnetii*. Restriction fragment-length polymorphism has been demonstrated and thus becomes a convenient marker for strain identification. A library has been constructed from the chromosomal DNA of Nine Mile phase I in a cosmid vector. The cloned DNA accounts for about 8000 kilobases (kb), which ensures a 95% chance for representation of any particular gene. During or subsequent to the spontaneous transition of Nine Mile phase I to phase II, a deletion of DNA has arisen. A Hae III fragment involved in the deletion was utilized to screen the cosmid bank. Hybridization analysis reveals that the clone includes the complete deletion (19 kb).

Plasmid DNAs have been extracted from a number of isolates and examined. The predominant type is associated with all laboratory strains currently examined. Two independent isolates from human clinical cases (chronic endocarditis) have an

identical plasmid profile, which can be distinguished from the previous prototype. Both types of plasmids have been cloned as single pieces for each in a cosmid vector. Crosshybridization of the plasmide reveals that an Eco RI fragment of the "cardiotropic" plasmid has little or no homology to any fragment of the laboratory-strain-plasmid prototype. This fragment has been subcloned in an expression vector; a polypeptide of about 50,000 kilodaltons is produced in an in vitro system programmed by the inserted DNA. Current investigations are determining whether this polypeptide will be diagnostic for strains with a potential of causing endocarditis in humans.

Progress:

Strain Identification Hae III digestions of chromosomal DNA followed by gel electrophoresis have been performed on six isolates of *C. burnetii*. The list of isolates and pertinent information are summarized in Table 1. Restriction fragment polymorphism (RFLP) was apparent; even within the 10 largest DNA fragments, four different patterns were identified. The DNA patterns of phase I American isolates were indistinguishable from each other, whereas the DNA patterns among the European isolates also appeared identical but were distinguishable from those of the American isolates. The restriction patterns of DNA from the two endocarditis isolates were identical, but differed the most from any other DNA. Five of the 10 largest fragments of the endocarditis strains had electrophoretic mobilities unique to those two strains.

Even though Nine Mile II is a spontaneous derivative from Nine Mile I, the Hae III patterns revealed that the largest fragment of phase I was absent in the phase II organisms. Preparative electroelution of that fragment (band 1) and subsequent radiolabeling in vitro showed that sequences represented by the 4.3-kb fragment also were missing. The sequences are, however, present in all other phase II organisms examined, and apparently the absence is associated specially only with Nine Mile phase II.

Construction of a Library A partial library of Nine Mile phase I chromosomal DNA was constructed previously (4), utilizing a γ-based vector. A complete bank was constructed utilizing pHC79, a cosmid-based vector (5). Chromosomal DNA from Nine Mile phase I and vector DNA were restricted with Bam HI and ligated in a 2:1 molar ratio. The ligation mixture was packaged in vitro with a γ-packaging extract and subsequently transfected into an *E. coli* host. Independent transductants were selected on the basis of ampicillin resistance contributed by the vector. About 200 such colonies were isolated. The DNAs from several independent colonies were purified and analyzed, confirming the presence of various large fragments inserted at the Bam HI site of the vector.

For DNA to be a substrate for the packaging reaction, it should range in size between 35 to 45 kb. Since the vector accounts for 6.5 kb, the size estimated for the library is 200 x 35 or 7000 kb. This size is five times the size of the genome of *C. burnetii* (6) and ensures a 95% chance for representation of any particular sequence (7).

Mapping of the Deletion of Nine Mile Phase II The Hae III fragment of Nine Mile I was hybridized directly to lysed colonies comprising the library. Three colonies scored positive and the DNAs from those three were purified and analyzed by Hae III digestion. Two of the three had a fragment that co-migrated with band 1 and also showed an identical Hae III restriction pattern. One of the DNAs was radiolabeled in vitro and used to probe the Hae III digests of chromosomal DNAs of various strains of *C. burnetii* (Southern blot). The hybridization profiles of Nine Mile phase I and II suggested that Hae III fragments, in addition to band 1, were absent in Nine Mile II chromosomal DNA. A restriction map of the cloned DNA was generated. The missing Hae III fragments mapped as a continuous 19-kb segment within the cloned 37-kb fragment. Thus, one can hypothesize that a deletion occurred during or subsequent to the phase transition (I τ II). The cloned DNA may control a (virulence) function that is required for phase I maintenance. Genetic and biochemical assays are being designed to delineate this function.

Cloning of Two Endogenous Plasmids of *C. burnetii*. A plasmid has been described that is present in Nine Mile phase I and II (8). The isolates that were available were screened for the presence of plasmids by preparative ethidium bromide-CsCl density gradients of lysates. Isolated plasmid (circular) DNAs thus were purified and analyzed by several restriction endonucleases. Most strains analyzed exhibited a plasmid that could not be distinguished from the one previously described. On the other hand, the two endocarditis isolates had an identical plasmid that was related but distinguishable from the predominant prototype.

Based on both chromosomal and plasmid profiles, it is possible that a strain exists that can cause a different spectrum of pathology. The plasmid certainly is a marker of this "cardiotropic" strain and may even contribute to the altered pathology. Both plasmid types are about 35 to 40 kb, which is an appropriate size for cloning in a cosmid. The plasmids were partially digested with Sal I, inserted into the Sal I site of pHC79, and transduced into *E. coli*. By screening the DNA of several colonies from each plasmid prototype, it was possible to isolate a clone of each that included all the restriction fragments of the native (uncloned) plasmid DNAs.

Subcloning of Cardiotropic Plasmid The plasmid is a marker that may be useful in distinguishing cardiotropic strains from others. One way to exploit this potential is to focus on a DNA segment or a polypeptide characteristic of a plasmid prototype. Both plasmids were radiolabeled and cross-hybridized to various restriction patterns of the other. An Eco RI fragment (5th largest) of the cardiotropic plasmid did not appear to be homologous to sequences in the other plasmids. An Eco RI digest of the cardiotropic plasmid was "shotgun" cloned into Puc 8. DNA from colonies was screened and a clone representing that fragment was isolated. The DNA of that fragment was retested by hybridization. It apparently does cross-hybridize to some extent (about 20% of homologous value). The heterologous hybridization is split between two different Eco RI fragments. Probably the hybridization occurs at either end of the fragment, and the central 80% is unique.

Puc 8 is an expression vector inducible with isopropyl β-thiogalactoside. The cloned DNA was analyzed for coding potential by programming a coupled in vitro transcription, translation system. Two prominently labeled polypeptides were produced: one was the β-lactamase coded by the vector portion of the molecule, and the other was a 50,000-dalton polypeptide characteristic of this clone only.

Current experiments are investigating whether serum from endocarditis patients will selectively cross-react with labeled polypeptide. Such a reagent could have significant diagnostic utility and could lead to a more aggressive drug regimen for patients with acute Q fever, who had been infected with a cardiotropic strain.

PRESENTATIONS:

1. **Vodkin, M. H., J. C. Williams, and E. H. Stephenson.** A function of *Coxiella burnetii* that excludes the 1059 (phasmid) DNA from a normally permissive *Escherichia coli* host strain. Presented, 8th Annual Mid-Atlantic Regional Extrachromosomal Genetic Elements Meeting, Virginia Beach, VA, Oct 1984.

2. **Vodkin, M. H.** Recombinant DNA vaccines. Presented, Dept. of Biology, University of South Carolina, Columbia, SC, Nov 1984.

3. **Vodkin, M. H., J. C. Williams, and E. H. Stephenson.** Deletion of chromosomal DNA fragment between phase I and phase II *Coxiella burnetii*. Presented, American Society of Rickettsiology, Laguna Beach, CA, Feb 1985.

4. **Vodkin, M. H., J. C. Williams, and E. H. Stephenson.** Deletion of chromosomal DNA fragment between phase I and phase II *Coxiella burnetii*. Presented, 85th Annual Meeting of the American Society of Microbiology, Las Vegas, NV, Mar 1985.

5. **Vodkin, M. H., J. C. Williams, and E. H. Stephenson.** Recognition of a causative agent of chronic endocarditis as a distinct strain of *Coxiella burnetii*. Presented, 54th Annual Genetics Society of America Meeting, Boston, MA, Aug 1985.

6. **Vodkin, M. H., K. Amano, J. C. Williams, and E. H. Stephenson.** Deletions corelate with altered virulence and lipopolysaccharide structure of *Coxiella burnetii*. Presented 9th Annual Mid-Atlantic Regional Extrachromosomal Genetic Elements Meeting, Virginia Beach, VA, Sep 1985.

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1. **Mikesell, P., and M. H. Vodkin.** 1985. Plasmids of *Bacillus anthracis*, pp. 52-55 *In* Microbiology 1985, American Society for Microbiology, Washington, DC.

2. **Vodkin, M. H., J. C. Williams, and E. H. Stephenson.** 1985. A function of *Coxiella burnetii* that excludes the 1059 (phasmid) DNA from a normally permissive *Escherichia coli* host strain. *Plasmid* 13:220-221.

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1. **Peacock, M. G., R. N. Philip, J. C. Williams, and R. S. Faulkner.** 1983. Serological evalatuion of Q fever in humans: enhanced phase I titers of immunoglobulins G and A are diagnostic for Q fever endocarditis. *Infect. Immun.* 41:1089-1098.

2. **Berman, S., R. B. Gochenour, J. P. Lowenthal, and A. S. Benenson.** 1981. Method for the production of a purified Q fever vaccine. *J. Bacteriol.* 81:794-799.

3. **Williams, J. C., and J. L. Cantrell.** 1982. Biological and immunological properties of *Coxiella burnetii* vaccine in C57BL/10 ScN endotoxin nonresponder mice. *Infect. Immun.* 35:1091-1102.

4. **Vodkin, M. H.** 1984. U. S. Army Medical Research Institute of Infectious Diseases Annual Progress Report, FY 1984.

5. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning - a laboratory manual. Cold Spring Harbor.

TABLE 1. *Coxiella burnetii* isolates.

Strain[a]	Geographic location	Source	Passage history[c]	Phase[d]
Nine Mile				
(CB9MIC7)	Montana[b]	Tick	1TP/307GP/1TC/2EP/1MP/2EP	I
(C9MIIC4)			1TP/304GP/90EP/1TC/4EP	II
Ohio (CBOI)	Ohio	Bovine milk	1BP/5EP/2GP/2EP	I
PAV (CBPAVI)	California	Human aortic valve	1HP/5EP	I
KAV	Oregon	Human Aortic	1HP/1GP/3EP	I
Henzerling (CBHENI)	Italy	Human blood clot	1HP/6GP/25EP/1GP/2EP	I
M44	Greece	Human blood	1HP/1GP/86EP/20 GP/50MP/5EP	II

[a]Strains are designated as *Coxiella burnetii* (CB). In addition they were identified considering the geographic location, the source of the isolated microorganism, and the passage history.
[b]This strain was isolated near Nine Mile Creek in western Montana.
[c]Passage history: GP, guinea pig; TC, tissue culture; EP, embryonated egg passage; HP, human passage; BP, bovine passage; MP, mouse passage; TP, tick passage.
[d]Phase determined by lipopolysaccharide chemotype.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 305755
2. DATE OF SUMMARY: 85 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 84 12 01
4. KIND OF SUMMARY: D. CHANGE
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	LA	139
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) In Vitro Effect of Hemorrhagic Fever Viruses on Endothelial Cells

12. SUBJECT AREAS 0613 Microbiology; 0605 Clinical Medicine

13. START DATE: 84 12
14. ESTIMATED COMPLETION DATE: 87 06
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE | EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE | d. AMOUNT
e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	1.0	147
86	1.0	220

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: USA Medical Research Institute of Infectious Diseases
b. ADDRESS (include zip code): Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL: Huxsoll, D L
d. TELEPHONE NUMBER (include area code): 301-663-2833

20. PERFORMING ORGANIZATION
a. NAME: Medical Division, USAMRIID
b. ADDRESS: Fort Detrick, MD 21701-5011
c. NAME OF PRINCIPAL INVESTIGATOR: Lewis R M
d. TELEPHONE NUMBER (include area code): 301-663-7655
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE: FIC
MILITARY/CIVILIAN APPLICATION: L

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Endothelial Cells; (U) Coagulation Factors; (U) Hemostasis; (U) Hemorrhagic Fever Viruses

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Devise an in vitro model to determine the pathogenesis of the hemostatic derangement produced by hemorrhagic fever viruses. This work will ultimately benefit military personnel exposed to these viruses either in endemic areas or because of their use in biological warfare.

24. (U) Endothelial cell cultures utilizing human, bovine, and hybrid cells will be infected with hemorrhagic fever viruses and the effects on these cells, particularly with regard to hemostatic functions, will be determined.

25. (U) Endothelial cell cultures from human umbilical veins and bovine aorta have been successfully established. A hybrid cell line formed by fusing fibroblast and endothelial parent cells is also maintained in culture. The endothelial and hybrid cells have been successfully infected with a number of hemorrhagic fever viruses, including Ebola, Marburg, Lassa, Rift Valley fever, Hantaan, and Junin. The infected cells continue to produce factor VIII antigen to the same degree as uninfected cells. Assays for other endothelial-produced coagulation factors have been adapted for use in these studies. These include assays for plasminogen activator, plasminogen activator inhibitor, thrombomodulin and prostacyclin.

BODY OF REPORT

PROJECT NO. 3A161101A91C: In-House Laboratory Independent Research

WORK UNIT NO. 91C-LA-139: In Vitro Effect of Hemorrhagic Fever Viruses on Endothelial Cells

PRINCIPAL INVESTIGATOR: Richard M. Lewis, Ph.D.

Background:

Hemorrhagic fever is a clinical syndrome which is often recognized in association with a number of viral diseases. These infections are a world health problem. Though not endemic to the United States, they do present a health risk to American travelers, especially military personnel who might be stationed in those areas where the disease is prevalent. In addition, many hemorrhagic fever viruses can be transmitted by aerosol and are thus potential biological weapons against non-immune American troops.

These diseases share the ability to cause drastic changes in the hemostatic mechanism. It is not clear whether these changes are associated with altered coagulation protein activity, impaired platelet function, or altered capillary reactivity. Pathologically, the changes in hemostasis appear widespread and not confined to specific organs.

The endothelium lines vessels throughout the entire vascular system. Endothelial cells, originally thought to form merely a non-thrombogenic surface, have been shown more recently to produce important molecules which function in coagulation, platelet aggregation, and capillary integrity. In addition, these cells respond to immune effector molecules and produce immunologically important products.

Alterations in the important control functions exerted by endothelial cells could result in drastic changes in the ability of the body to control bleeding. If viral infections associated with hemorrhagic fever produce such changes, this provides one explanation for the hemostatic impairment seen in these infections.

Summary:

Primary cultures of human endothelial cells are maintained in the Coagulation Laboratory on a routine basis. A human-derived cell line, EA926, which is biochemically similar to endothelial cells, is also maintained in culture. A number of hemorrhagic fever viruses have been shown to infect both cell types. Viruses studied include: Ebola, Marburg, Lassa, Rift Valley fever, Hantaan, and Junin. Endothelial and EA926 cells have been shown to exhibit factor VIII antigen on their surface during infection. Assays for endothelial cell-produced coagulation factors have been adapted for use in tissue culture fluids and are presently being applied. These include: plasminogen activator (ELISA, chromogenic, fibrinolytic activity), plasminogen activator inhibitor, factor VIII antigen, protein C activating activity (thrombomodulin), and prostacyclin.

Progress:

In order to determine if viral infection alters endothelial cell function, methodology was instituted to isolate cells from human umbilical veins and maintain them in culture. This is now done routinely with results similar to those reported in the literature. An endothelial cell line, EA926, is also routinely cultured and used as a model for endothelial cells. Both human endothelial cells and EA926 cells have been used for infectivity studies with a number of hemorrhagic fever viruses. Using fluorescent antibody techniques, we can detect viral antigen in these cells following infection with Ebola, Marburg, Lassa, Hantaan, and Rift Valley fever viruses. In addition, infection with Marburg, Ebola, Lassa, Rift Valley fever, Junin, and Hantaan viruses were productive, as demonstrated by increases in viral plaque-forming units when measured over time.

Factor VIII antigen, an important protein for platelet binding to endothelium, is also a marker for endothelial cells. With fluorescent antibody techniques, the presence of factor VIII antigen can be detected in infected endothelial cells in a intensity similar to that in uninfected cells.

Pathology studies from E. D. Johnson's laboratory suggest alterations in fibrinolysis during Ebola infections. Because endothelial cells produce plasminogen activator and its inhibitor, the potential exists for fibrinolytic defects arising from viral alterations in these cells. Thus, plasminogen activator PLAS studies have been initiated to measure PLAS antigen, PLAS fibrinolytic activity, PLAS amidolytic activity, and PLAS inhibitor activity in Ebola plasma and in Ebola-infected, endothelial cells.

PUBLICATIONS: None

PRESENTATIONS:

1. **Lewis R. M., E. D. Johnson, P. B. Jahrling, C. -J. Edgell, T. M. Cosgriff, and C. J. Peters.** In vitro infection of endothelial cells by Ebola, Lassa, and Marburg viruses. Presented at the Annual Meeting of the American Society of Microbiology, Las Vegas, Nevada, March 1985.

2. **Lewis R., L. Hodgson, and T. Cosgriff.** The effect of T-2 mycotoxin on tissue factor production by human monocytes and U937 cells. Presented at the Xth International Conference on Thrombosis and Haemostasis, San Diego, California, July 1985.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 306147	85 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
85 04 04	D. CHANGE	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	LA	140
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Cloning of Snake Toxin Genes for Novel Vaccine Development

12. SUBJECT AREAS 0605 Clinical Medicine; 1503 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 12	88 01	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		85	1.0	157
c. TYPE	d. AMOUNT	86	1.0	240
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathology Division, USAMRIID
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L	c. NAME OF PRINCIPAL INVESTIGATOR Smith, L A
d. TELEPHONE NUMBER (include area code) 301-663-2833	d. TELEPHONE NUMBER (include area code) 301-663-7211
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR (if available)
MILITARY/CIVILIAN APPLICATION L	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) BW Defense; (U) Cloning; (U) Vaccines; (U) Snake; (U) Toxin; (U) Genes

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objectives of this research are to clone, sequence, and express the genes coding for the toxic factors from cobra snake venom. The knowledge gained is expected to lead directly to the development of a novel vaccine for these snake toxins.

24. (U) The initial approach involves (1) obtaining the glands from the cobra snake *Naja naja atra*, (2) isolating and purifying the DNA from these glands, (3) digesting the genomic DNA with restriction enzymes, (4) inserting these fragments into a suitable vector (plasmid), (5) analyzing the genomic library with oligonucleotide probes homologous to toxic gene sequences, (6) examining the products of the clones immunologically, (7) manipulating the cloned genes into producing maximum quantities of products for vaccine development, and (8) utilizing site-specific mutagenesis at the molecular level to produce non-toxic antigenic proteins.

25. (U) In 1985, we report (1) the purification to apparent homogeneity of cobratoxin, cardiotoxin and phospholipase A_2 from the venom of *Naja naja atra*, (2) the production of affinity-purified rabbit antibodies against these 3 toxins as well as the neurotoxin and cardiotoxin from *Naja mossambica* and the neurotoxin from *Naja nigricollis*, and (3) the generation of 130 recombinant DNA clones having cobra DNA inserts. These clones are presently being analyzed to see if they contain the genes for cobratoxin, cardiotoxin, or phospholipase A_2.

BODY OF REPORT

PROJECT NO: 3A161101A91C: In-House Laboratory Independent Research

WORK UNIT NO: 91C-LA-140: Cloning of Snake Toxin Genes for Novel Vaccine Development

PRINCIPAL INVESTIGATOR: Leonard A. Smith, Ph.D.

Background:

There exist in nature a wide variety of poisonous animals that produce many different types of toxins. Animals such as spiders, scorpions, beetles, insects, snakes, etc., have venom glands that produce a mixture of different compounds, some of which are acutely deleterious to man. Limited studies have been made on various venoms, but, as yet, little is known concerning the pharmacology, chemical properties, and mechanism of action of the toxic components of animal venoms. Of these, snake venoms have been studied to the greatest extent.

Venoms of snakes belonging to the families *Elapidae* (cobra, krait, coral, mamba, tiger, copperhead, taipan, black, death adder, etc.) and *Hydrophidae* (many species of marine snakes) are extremely toxic and produce flaccid paralysis and respiratory failure in animals (6,4). Although this toxicity is based on an amalgamation of constituents, one protein family stands out in having very pronounced pharmacological activities. Some members of this family are potent neurotoxins while others produce cardiotoxic and cytolytic effects. Since the neurotoxins and cardiotoxins in snake venom can be readily separated from the other toxins, enzymes, and peptides by gel filtration and ion-exchange chromatography, much progress has been achieved in the area of structure-function relationships.

Studies on the primary structure, structure-function relationships, phylogenetics, and immunochemical properties of snake venom toxins have led to the classification of the toxins into three groups: short neurotoxin, long neurotoxin, and cardiotoxin. The short neurotoxins contain 60 to 62 amino acid residues in a single peptide chain and are cross-linked by four disulfide bridges (7). The long neurotoxins are comprised of 71 to 74 residues and have five disulfide bridges (7). Besides the neurotoxins, elapid venoms contain cardiotoxins (cytotoxins and/or direct lytic factors) which are homologous to and phylogenetically close to the neurotoxins. Cardiotoxins comprise 60 to 61 amino acid residues cross-linked by four disulfide bridges.

The mode of action of elapid neurotoxins is to produce an anti-depolarizing neuromuscular (N-M) block by acting on the post-junctional membrane of the motor endplate. Unlike the effect of crude venoms, the N-M blockage by purified *Naja naja atra* neurotoxin can be reversed by either neostigmine or repeated washing. However, some neurotoxins (e.g. *Naja naja naja*) bind to cholinergic receptors and block N-M transmission irreversibly. The LD_{50} of cobra neurotoxin is $\simeq 50$ to 150 μg/kgm body weight (3,5), which is about six times more lethal than the crude venom. Cardiotoxins affect different types of cells (e.g. skeletal muscle, smooth muscle, peripheral nerve cells), causing irreversible depolarization of the cell membrane and consequently impairing both the function and structure of cells (5). The LD_{50} of purified cardiotoxin injected i.p. in mice is 2.8 mg/kgm body weight (3,5).

Although there have been extensive pharmacological and protein chemical studies on the snake toxins, genetic investigation on toxin genes has not been an active research area. It is the intent of this ILIR to utilize recombinant DNA technology to clone and express the genes coding for the snake toxins.

The initial approach to be taken with the cloning of the snake toxin genes involve (1) obtaining the glands from the cobra snake (*Naja naja atra*), (2) isolating and purifying the DNA with restriction enzymes, (4) inserting these fragments into a suitable vector (plasmid), (5) analyzing the genomic library with oligonucleotide probes homologous to toxin gene sequences, (6) examining the products of the clones from the genomic library immunologically, (7) manipulating the cloned genes into producing adequate quantities of products, and (8) utilizing site-specific mutagenesis at the molecular level to produce nontoxic proteins which would be cross-reacting immunologically but have a high safety value. As stated earlier, there exist many different types of lethal toxins and the cloning and expression of the cobra toxin genes will hopefully provide a model system for future vaccine development for other low molecular weight and potential BW toxins.

Summary:

In 1985, we report (1) the purification to apparent homogeneity of three snake toxins: cobratoxin, cardiotoxin, and phospholipase A_2, from the venom of *Naja naja atra*, (2) The production of affinity-purified rabbit antibodies against cobratoxin, phospholipase A_2, and three cardiotoxins from *Naja naja atra* as well as the neurotoxin and cardiotoxin from *Naja mossambica pallida* and the neurotoxin from *Naja nigricollis*, and (3) the generation of 130 recombinant DNA clones having cobra DNA inserts. These clones are presently being analyzed to see if they contain the genes for cobratoxin, cardiotoxin, or phospholipase A_2.

Progress:

In order to effect the cloning research, specific materials were required. These materials included DNA and RNA from snake glands, oligonucleotide probes, homogeneous snake toxins, and affinity-purified antibodies to the purified snake toxins. The snake glands from *Naja naja atra* were obtained from Dr. Y. Sawai of the Japanese Snake Institute in Gunma, Japan. Although the glands were thawed upon arrival from Japan, high molecular weight DNA was still isolated by a phenol extraction of the minced tissue. Agarose gel electrophoresis of the DNA revealed the DNA to be degraded but still usable for our cloning purposes. The oligonucleotide probes to the α-cobratoxin, cardiotoxin, and phospholipase A_2 were synthesized by published amino acid sequences for these proteins.

Cobra snake venom was commercially obtained from Sigma Chemical Company and the three toxins of interest (α-cobratoxin, cardiotoxin and phospholipase A_2) were purified to apparent homogeneity by successful runs over a preparative FPLC Mono-S column. Analysis by SDS-PAGE, and, in some cases, amino acid sequencing showed the three toxins to be homogeneous. These toxins from *Naja naja atra* as well as purified neurotoxin and cardiotoxin from *Naja mossambica pallida* and purified neurotoxin from *Naja nigricollis* were used to immunize 19 New Zealand rabbits. The rabbits were injected initially with sub-lethal doses of toxin in the presence of Complete Freunds Adjuvant. They were boosted twice, three weeks apart, in the present of Incomplete Freunds Adjuvant. On the second week after their second boost, they were bled from the ear (50 ml blood/rabbit was obtained). Sera from the pre-immunized and immunized rabbits were analyzed for antibodies that cross-reacted with *E. coli* proteins. Both sera did cross-react with *E. coli* extracts. Because we are cloning into *E. coli*, it was necessary to separate the *E. coli* antibodies from the snake antibodies so we could use the snake antibodies as reagents for analyzing our cloned products. Two affinity columns were prepared by covalently attaching either α-cobratoxin or cardiotoxin to activated Sepharose. The sera from the immunized rabbits were passed over the affinity columns and the snake antibodies were purified from the *E. coli* antibodies. At this stage, having the reagents required to analyze recombinant clones, we began our cloning experiments.

The snake DNA was partially digested with the restriction enzyme EcoRl and cloned into the EcoRl site of the plasmid vector pUC8. Plasmid pUC8 is a small (2.7Kb) plasmid containing the ampicillin resistance gene from pBr322 as well as a DNA segment from the lac gene encoding a short polypeptide which, in suitable hosts (eg. JM101 and JM103), will allow the formation of functional β-galactosidase molecules in cells which could not otherwise produce such molecules. pUC8-containing colonies can thus be distinguished by their blue color on appropriate indicator (X-gal) plates. The lac region of the plasmid contains a polylinker with unique cloning sites for nine enzymes. Insertion of DNA into any one of these sites causes disruption of the peptide conferring β-galactosidase activity, so that colonies containing recombinant plasmids can be distinguished from those containing pUC8 itself by their white color on X-gal plates. Thus, EcoRl-restricted cobra DNA was inserted into pUC8 and transformed into JM103. Colonies that grew on LB agar containing ampicillin were analyzed for β-galactosidase activity using the X-gal substrate. We obtained 130 colonies that contained cobra DNA inserts. Individual colonies were grown up on nitrocellulose filters, lysed with chloroform, and hybridized with ^{32}P-oligonucleotide probe to either cardiotoxin or cobratoxin. All of the colonies hybridized with the oligonucleotide probes. The reason for this is that the oligonucleotide probes reacted with the pUC8 DNA under the conditions of the hybridization experiment. We then grew up each of the clones in 20 ml of L-Broth containing ampicillin, isolated the plasmid DNA by an alkaline extraction, restricted the DNA with EcoRl, and analyzed the resulting fragments on agarose gel electrophoresis. The DNA fragments were transferred to nitrocellulose filters. The cobra snake venom DNA fragments, now separated from the pUC8 vector, are awaiting hybridization with the probes.

The clones hybridizing to the oligonucleotide probes will be examined for the size of the insert and for the product(s) that may be expressed. At this point, identification of expressed products is a primary task. Immunological methods, such as the one of Broom and Gilbert (1), and Western blot analysis (2) will be performed by using the affinity-purified antibodies to the snake toxins. The latter analysis will reveal whether or not we have cloned and are expressing the toxin genes in *E. coli*.

PRESENTATIONS: None.

PUBLICATIONS: None.

LITERATURE CITED

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3. **Dufton, M. J., and R. C. Hider.** 1983. *Crit. Rev. Biochem.* 14:113.

4. **Lee, C. Y.** 1979. Snake venoms. *In* Handbook of experimental pharmacology. 52. Springer-Verlag, Berlin.

5. **Lee, C. Y.** 1972. *Ann. Rev. Pharmacol.* 12:265.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	DA 307174	2. DATE OF SUMMARY	85 10 01	REPORT CONTROL SYMBOL	DD-DR&E(AR) 636	
3. DATE PREV SUM'RY	85 04 23	4. KIND OF SUMMARY	D. CHANGE	5. SUMMARY SCTY	U	
6. WORK SECURITY	U	7. REGRADING		8. DISB'N INSTR'N	CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	141
b. CONTRIBUTING				
c. CONTRIBUTING	NONE			

11. TITLE (Precede with Security Classification Code) Investigation of the Feasibility of Producing a Broadly Cross-Protective Alphavirus Vaccine Using Recombinant DNA Technology

12. SUBJECT AREAS
0605 Clinical Medicine; 1503 Defense; 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
85 04	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE | d. AMOUNT
e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
85	1.0	67
86	0.3	250

19. RESPONSIBLE DOD ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases
b. ADDRESS (include zip code) Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Huxsoll, D L
d. TELEPHONE NUMBER (include area code) 301-663-2833

20. PERFORMING ORGANIZATION
a. NAME Virology Division, USAMRIID
b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF PRINCIPAL INVESTIGATOR Dalrymple, J M
d. TELEPHONE NUMBER (include area code) 301-663-2665
f. NAME OF ASSOCIATE INVESTIGATOR (if available) Markiewicz, P
g. NAME OF ASSOCIATE INVESTIGATOR (if available) Hasty, S

21. GENERAL USE FIC
MILITARY/CIVILIAN APPLICATION: L

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Medicine; (U) Vaccines; (U) Alphavirus; (U) Defense; (U) Recombinant DNA; (U) Monoclonal Antibodies; (U) Cross-protection

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objectives of this research are to: a) develop the ability to rapidly identify DNA sequences in structural protein coding genes of alphaviruses and related virus variants, b) define the major antigenic determinants of alphavirus pathogens of military medical importance and prepare diagnostic reagents, c) evaluate the feasibility of incorporating different viral genes or gene segments into a vaccine virus vector, and d) evaluate the potential prophylactic value of broadly reactive alphavirus immunogens derived from recombinant DNA technology.

24. (U) Investigate alphaviruses that are antigenically closely related. Lymphocyte hybridomas will be prepared, and specific antibodies to determinants associated with neutralization and/or animal protection will be emphasized. Genes coding for the structural proteins of selected alphaviruses will be cloned and sequenced. Sequences conserved among alphaviruses will be used for rapid sequence analysis of different isolates and virus mutations. Sequences will be inserted into vaccinia virus as a vector for the expression of potentially protective immunogens.

25. (U) 8504-8509-A series of 11 different alphavirus isolates have been propagated to high titers, plaque-purified to provide genetically homogeneous populations, and polyclonal hyperimmune antisera prepared to each. Antigenic cross-reactivity has been evaluated by radioimmune assay and plaque reduction neutralization testing. Virus purification, nucleic acid extraction, and RNA analysis are being performed preparatory to cloning.

BODY OF REPORT

PROJECT NO. 3A161101A91C: In-House Laboratory Independent Research

WORK UNIT NO. 91C-00-141: Investigation of the Feasibility of Producing a Broadly Cross-Protective Alphavirus Vaccine Using Recombinant DNA Technology

PRINCIPAL INVESTIGATOR: J. M. Dalrymple, Ph.D.

ASSOCIATE INVESTIGATORS: P. Markiewicz, CPT, Ph.D.
S. Hasty

Background

Conventional methodology that leads to protection of humans against virus infections requires the development of a live, attenuated or inactivated vaccine for each individual virus posing a threat to U.S. military personnel. However, the number of different viral pathogens, strains, and variants clearly demonstrates the complexity of such an undertaking. The development of recombinant DNA technology has provided a mechanism for dissecting viral gene products and examining their individual pathogenic effects and their potential for use in immunization and protection against virus-induced disease. If the individual genes coding for protective immunogens could be identified, it should be possible theoretically to construct a vaccine, capable of broad-spectrum protection, containing multiple genes from a variety of human pathogens.

Summary

A series of 11 different alphavirus isolates have been propagated to high titer, plaque-purified to provide genetically homogeneous populations, and polyclonal, hyperimmune antisera prepared against each virus. Antigenic cross-reactivity of each strain has been evaluated by radioimmune assay (RIA) and plaque-reduction neutralization (PRN). Virus purification, nucleic acid extraction, and RNA analyses are being performed before cloning of antigenic epitopes is done.

Progress

The approach to developing a new vaccine strategy begins with the selection of a group of closely related virus pathogens and the identification and characterization of their antigenic determinants, which are important in inducing protection against disease. The subsequent identification of the specific genes responsible for the expression of these protein antigens will require the recombinant DNA technologies of cloning and sequencing. Feasibility will be demonstrated if these genes can be isolated as DNA clones and inserted into a functional vector virus, such as vaccinia, that can be used to actively immunize experimental animals against more than a single virus.

A series of 11 different alphaviruses have been propagated to high titer, plaque-purified to provide genetically homogeneous populations. Stock virus suspensions have been propagated and characterized. Polyclonal, hyperimmune antisera have been prepared to each of the viruses; their antigenic cross-reactivities have been compared by neutralization and RIA. Lymphocyte hybridomas have been generated to produce monoclonal antibodies for two of the 11 virus strains. Methods for virus purification have been developed, and preliminary experiments of viral RNA have been completed, suggesting that RNA can be obtained in quantities sufficient for gene cloning experiments.

This is an extremely ambitious undertaking, that, if successful, will revolutionize the approach to vaccine development. The probability of success cannot be determined at this time. The study should require a maximum of two to three years in the ILIR program.

PRESENTATIONS: None

PUBLICATIONS: None

Appendix A
PUBLICATIONS BY
UNITED STATES ARMY MEDICAL RESEARCH INSTITUTE FOR INFECTIOUS DISEASES
FY 85

Allured, V.S., L.M. Case, S.H. Leppla, and D.B. McKay. 1985. Crystallization of the protective antigen protein of *Bacillus anthracis*. *J. Biol. Chem.* 260:5012-5013.

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APPENDIX B

CONTRACTS, GRANTS, MIPRS, and PURCHASE ORDERS IN EFFECT

UNITED STATES ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FY 85

CONTRACT NUMBER	TITLE, INVESTIGATOR, INSTITUTION
DAMD-17-84-C-4015	Epizootiology of Hantaan and Related Viruses in Baltimore. K.V. Shah, Johns Hopkins University, Baltimore, MD.
DAMD-17-83-C-3013	Synthetic Peptide Vaccines for the Control of Arenavirus Infection. M.J. Buchmeier, Scripps Clinic and Research Foundation, La Jolla, CA.
DAMD-17-84-C-4146	The Use of Resin Bound Monoclonal Antibody in the Purification of the Various Components in Anthrax Vaccines. J.W. Burnett, University of Maryland, Baltimore, MD.
DAMD-17-85-C-5208	Trichothecenes Mycotoxin Studies. P.M. Newberne, Massachusetts Institute of Technology, Cambridge, MA.
DAMD-17-84-C-4156	Freshwater Cyanobacteria (Blue-Green Algae) Toxins: Isolation and Characterization. W.W. Carmichael, Wright State University, Dayton, OH.
DAMD-17-84-C-4130	Molecular Basis of Paralytic Neurotoxin Action on Voltage-Sensitive Sodium Channels. W.A. Catterall, University of Washington, Seattle, WA.
DAMD-17-82-C-2021	Studies on the Immunochemical Techniques for Detection of Selected Fungal and Dinoflagellate Toxins. F.S. Chu, University of Wisconsin, Madison, WI.
DAMD-17-84-C-4046	Research and Development of Human and Primate Antibodies for Immunotherapy of Viral Hemorrhagic Fever Infections. R.M. Condie, University of Minnesota, Minnepolis, MN.
DAMD-17-84-C-4245	Study of Antigenic Structures of Botulinum Neurotoxin. B. DasGupta, University of Wisconsin, Madison, WI.
PO-84-PP-4861	The Role of Plasmids and Bacteriophages in Toxigenicity of *Clostridium botulinum* and Characterization of Converting Bacteriophages. M.W. Eklund, US Department of Commerce, Seattle, WA.
DAMD-17-84-C-4139	Potential Vaccine for Anthrax. R.J. Doyle, Louisville Foundation, Inc., Louisville, KY.
MIPR-83-MM-3501	Acute T-2 Intoxication: Physiologic Consequences and New Therapeutic Approaches. G.Z. Feuerstein, Uniformed Services University, Bethesda, MD.

DAMD-17-82-C-2237	Structure and Expression of Genes for Flavivirus Immunogens. M.J. Fournier, University of Massachusetts, Amherst, MA.
DAMD-17-79-C-9024	Lassa Fever Immune Plasma. J.D. Frame, Columbia University of Heal, New York, NY.
DAMD-17-84-C-4144	Selective Targeting of Antiviral Immunomodulating Agents in the Treatment of Arenavirus Infections. J.D. Gangemi, University of South Carolina, Columbia, SC.
PO-84-PP-4854	Expression of Immunogenic Virus Proteins in Eukaryotic Vector Systems. J. Hay, Uniformed Services University, Bethesda, MD.
DAMD-17-82-C-2240	Trichothecene Mycotoxins: Preparation, Analysis, and Chemical Reactivity. B.B. Jarvis, University of Maryland, College Park, MD.
PO-84-PP-4828	Kinetics of Percutaneous Absorption of Mycotoxins in Vitro. B.M. Wallner, US Department of Agriculture, Athens, GA.
DAMD-17-83-G-9563	Effects of Trichothecenes on Cardiac Cell Electrical Function. W.T. Woods, University of Alabama, Birmingham, AL.
DAMD-17-83-G-9565	Studies of Biological and Molecular Basis of the Inhibition of Activity of Phagocytic Cells by Anthrax Toxin. G.G. Wright, University of Virginia, Charlottesville, VA.
DAMD-17-82-C-2235	Trichothecenes Mycotoxin Studies. P.M. Newberne, Massachusetts Institute of Technology, Cambridge, MA.
DAMD-17-79-C-9046	The Synthesis and Study of New Ribavirin Derivatives and Related Nucleoside Azole Carboxamide as Agents Active Against RNA Viruses. R.K. Robins, Brigham Young University, Provo, UT.
DAMD-17-83-C-3114	Mechanism of Action of Presynaptic Neurotoxins. T.B. Rogers, Maryland University School of Medicine, Baltimore, MD.
DAMD-17-84-C-4003	Function of Mosquito Saliva in Delivery of Pathogens. P.A. Rossignol, Harvard University, Cambridge, MA.
DAMD-17-83-C-3020	Approaches to the Detoxication of Mycotoxins Using Glutathione Precursors and Analogs. A. Meister, Cornell University Medical College, New York, NY.
DAMD-17-82-C-2113	Metabolic Products, Mass Spectral Analyses and Synthesis of Toxic Trichothecenes. C.J. Mirocha, University of Minnesota, St. Paul, MN.
DAMD-17-82-C-2004	Mechanisms of Bunyavirus Virulence; a Genetic Approach. N. Nathanson, University of Pennsylvania, Philadelphia, PA.
DAMD-17-85-C-5008	The Synthesis of Radiolabelled Mycotoxins. G.A. Kraus, Iowa State University, Ames, IA.

DAMD-17-84-G-4016	Hemorrhagic Fever with Renal Syndrome (Korean Hemorrhagic Fever). H-W. Lee, Korean University Medical College, Seoul, Korea.
PO-85-PP-5825	Pathophysiology of Peptide Toxins of *M. aeruginosa* and *A. phylloides*. W.H. Admas, Brookhaven National Laboratory, Upton, NY.
DAMD-17-85-G-5006	Seroepidemiological Survey for Congo-Crimean Hemmorrhagic Fever and Hantaan Virus. A. Antoniadis, University of Thessaloniki Aristolean, Thessaloniki, Greece.
DAMD-17-85-C-5171	Characterization of the *P. brevis* Polyether Neurotoxin Binding Component in Excitable Membranes. D. Baden, University of Miami, Coral Gables, FL.
DAMD-17-85-C-5236	A Multi Disciplinary Study of Ciguatoxin and Related Low Molecular Weight Toxins from Marine Sources. L.E. Bailey, University of AT Manda Hawaii, Honolulu, HI.
DAMD-17-85-C-5241	Pathophysiology and Toxicokinetic Studies of Blue-Green Toxication in the Swine Model. V. Beasley, University of Illinois, Urbana, IL.
DAMD-17-82-C-2179	Toxicologic and Analytical Studies with T-2 and Related Trichothecene Mycotoxins. W.B. Buck, The University of Illinois, Urbana, IL.
DAMD-17-85-C-5224	Diagnosis and Management of Trichothecene Toxicosis in the Swine Model. W.B. Buck, University of Illinois, Urbana, IL.
DAMD-17-85-C-5202	Preparation and Characterization of Antiparalytic Shellfish Poison Poly and Monoclonal Antibodies for Development of Identification and Prophylaxis/Therapy Techniques. R.E. Carlson, Bio-Metric Systems, Inc., Eden Prairie, MN.
DAMD-17-85-C-5211	Feasibility of a Clinical Trial on the Use of Ribavirin in Epidemic Hemorrhagic Fever in China. T-W. Chang, New England Medical Center, Boston, MA.
DAMD-17-85-C-5249	Immunologic Approach to the Identification and Development of Vaccines to Various Toxins. T. Chanh, Georgetown University Medical, Washington, DC.
DAMD-17-85-C-5266	Human Hybridomas for Exotic Antigens. M. Cohn, The Salk Institute for Biology, La Jolla, CA.
DAMD-17-85-C-5226	Rift Valley Fever Virus: Molecular Biologic Studies of the M Segment RNA for Application in Disease Prevention. M. Collett, Molecular Genetics, Inc., Minnetonka, MN.
DAMD-17-85-C-5192	Toxin Photoaffinity Probes. D.S. Watt, University of Kentucky, Lexington, KY.

DAMD-17-85-G-5030	Engineered Organisms in the Environment: Scientific Issues. R.D. Watkins, American Society For Microbiology, Washington, DC.
MIPR-85-MM-5511	Preparation and Structural Analysis of Toxins, and Modeling of Toxins, Antibody Binding Sites and Antiviral Drugs. K.B. Ward, Naval Research Laboratory, Washington, DC.
DAMD-17-85-C-5002	Analysis of Trichothecene Mycotoxins by Combined HPLC-MS. P. and K. Vouros, Northeastern University, Boston, MA.
DAMD-17-85-C-5212	Genetic and Physiological Studies of *Bacillus anthracis* Related to Development of an Improved Vaccine. C. Thorne, University of Massachusett, Amherst, MA.
DAMD-17-85-C-5023	Studies of the Biology of Phleboviruses in Sandflies. R. Tesh, Yale University, New Haven, CT.
DAMD-17-85-C-5199	Identification and Selective Acquisition of Chemicals and Drugs for Antiviral Chemotherapy. E.L. Stephen, Technassociates, Inc., Rockvill, MD.
PO-85-PP-5830	Development and Evaluation of Supercritical Fluid Chromatography (SFC) and SFC-MASS Spectrometry for Analysis of Trichothecenes, Marine Toxins and Neurotoxins. R.D. Smith, Battelle Memorial Institute, Richland, WA.
PO-85-PP-5829	Development of Capillary Zone Electrophoresis/Mass Spectrometry for Analysis of Marine Toxins. R.D. Smith, Battelle Memorial Institute, Richland, WA.
DAMD-17-85-C-5285	Therapeutic Approaches to the Treatment of Botulism. L. Simpson, Jefferson Medical College, Philadelphia, PA.
DAMD-17-85-C-5276	Development of Systems for Delivery of Antiviral Drugs. W. Shannon, Southern Research Institute, Birmingham, AL.
DAMD-17-85-C-5071	Chemical Preparation Laboratory for IND Candidate Compounds. E. Schubert. Pharm-Eco Laboratories, Inc., Simi Valley, CA.
DAMD-17-85-C-5282	Alphavirus Epitopes of Vaccine Relevance. A.L. Schmaljohn, University of Maryland, Baltimore, MD.
DAMD-17-85-C-5167	Use of Recombinant DNA Techniques for the Production of a More Effective Anthrax Vaccine. D. Robertson, Brigham Young University, Provo, UT.
DAMD-17-85-C-5274	*Coxiella burnetii* Lipopolysaccharides: Structural Characterization, Chemicalynthesis and Immunogen Development. V. Reinhold, Harvard School of Public Health, Boston, MA.
DAMD-17-85-C-5232	Genetically-Engineered Poxviruses and the Construction of Live Recombinant Vaccines. E. Paoletti, Laboratory of Immunology, Albany, NY.

DAMD-17-85-C-5204	Metabolism, Mass Spectral Analysis and Mode of Action of Trichothecene Mycotoxins. C. Mirocha, University of Minnesota, St. Paul, MN.
DAMD-17-85-G-5005	Gordon Research Conference on *Fusarium* Mycotoxins. C.J. Mirocha, University of Minnesota, St. Paul, MN.
MIPR-85-MM-5512	Development of Vaccine to Mycotoxin T-2. V. Manohar, Immuquest Labs, Rockville, MD.
DAMD-17-85-C-5119	Resident Research Associateship Program (postdoctoral and senior postdoctoral). H.W. Lucien, National Academy of Sciences, Washington, DC.
PO-85-PP-5844	Immunologic Studies on the Effect of Trichothecenes. D. Lubaroff, Veterans Administration Medical Center, Iowa City, IA.
DAMD-17-85-C-5283	Functional Consequences of Chemical Modification of the Saxitoxin Binding Site on Neuronal Sodium Channels. B.K. Krueger, University of Maryland, Baltimore, MD.
PO-85-PP-5872	Structure Elucidation of Algal Toxins. T. Krishnamurthy, Chemical Research Laboratory Aberdeen, MD.
DAMD-17-85-C-5280	Mechanism of Action of Tetanus Toxin. M. Klempner, New England Medical Center Hospital, Boston, MA.
DAMD-17-85-G-5020	Tetrodotoxin, Saxitoxin, and the Molecular Biology of the Sodium Channel. C.Y. Kao, The New York Academy of Science, New York, NY.
DAMD-17-85-C-5129	Preparation of Radio Labelled Macrocyclic Trichothecenes, Simple Trichothecenes for Generation of Generic Antibodies. B.B. Jarvis, University of Maryland, College Park, MD.
DAMD-17-85-C-5054	*Bordetella* Extracytoplasmic Adenylate Cyclase: Structural and Functional Analogies with *Bacillus anthracis* Edema Factor Adenylate Cyclase. E.L. Hewlett, University of Virginia, Charlottesville, VA.
DAMD-17-85-C-5047	Analysis of a Pilot Study of Acute and Subchronic Toxicology of T-2 Toxin in Cynomologus Monkeys. C. Hassler, Battelle Columbus Laboratories, Columbus, OH.
PO-85-PP-5804	Investigate Immunoassay Development for Field Detection of Saxitoxin and Gonyautoxins. R.A. Elston, US Department of Energy, Richland, WA.
PO-85-PP-5833	Synthesis of Prodrugs of L-Cysteine as Prophylactic Agents Against Low Molecular Weight Toxins. H.T. Nagasawa. Veterans Medical Center, Minneapolis, MN.

APPENDIX C
PRESENTATIONS
(Abstracts)
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Huggins, J.W., G.R. Kim, and K.T. McKee, Jr. Ribavirin therapy of hemorrhagic fever with renal syndrome (HFRS) in the Hantaan virus suckling mouse model. Presented, Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, November 1984.

Luscri, B.J., P.G. Canonico, and J.W. Huggins. Synergistic antiviral interactions of alpha and gamma human interferons in vitro. Presented, Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, November 1984.

Friedlander, A.M. Microbial capsular polyanions alter the density of macrophage lysosomes. Presented, Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, November 1984.

Martin, M.B., and P.G. Canonico. The antiviral activity of 2-amino-1,3,4-selenadiazole and its effects on nucleic acid metabolism. Presented, Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, November 1984.

Turell, M.J., C.A. Rossi, and C.L. Bailey. Effect of extrinsic incubation temperature on the ability of *Aedes taeniorhynchus* and *Culex pipiens* to transmit Rift Valley fever virus. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

Turell, M.J., and C.L. Bailey. Failure of Rift Valley fever virus-inoculated larvae to successfully emerge as adults. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

Gargan, T.P.,II, G.G. Clark, D.J. Dohm, and C.L. Bailey. Experimental transmission of Rift Valley fever virus by North American mosquitoes. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

Faran, M.E., R.F. Tammariello, R.G. Routier, and C.L. Bailey. Spatial and temporal dissemination of Rift Valley fever virus in orally infected *Culex pipiens*. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

Tammariello, R.F., M.E. Faran, J. Meegan, and C.L. Bailey. Application of ELISA for detection of Rift Valley fever virus antigen in mosquitoes. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December, 1984.

Kenyon, R.H., and C.J. Peters. Strain heterogeneity of Junin virus. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

Scott, G.H., and E.H. Stephenson. Susceptibility of strains of inbred mice to *Coxiella burnetii* infection. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

Snyder, C.E., Jr., and J.C. Williams. Isolation and characterization of proteinaceous antigens of *Coxiella burnetii*. Presented, American Society of Tropical Medicine and Hygiene, Baltimore, MD, December 1984.

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GLOSSARY

ABC	Avidin-biotin-peroxidase
ACTH	Adrenocorticotrophic hormone
AFB	Airforce base
AFP	Alphafetoprotein
AG-B	African green monkey - Barbados substrain
AHF	Argentine hemorrhagic fever
A/J	Mouse strain
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APP	Avidin-polyalkaline-phosphate
ATP	Adenosine triphosphate
BALB/c	Mouse strain
BAW	Bronchoalveolar washing
BHF	Bolivian hemorrhagic fever
BHT	Butylated hydroxytoluene
Botx	Botulinum toxin
BP	Bovine passage
BPL	Beta propiolactone
BSA	Bovine serum albumin
BW	Biological warfare
C57BL/6	Mouse strain
CAR	Central African Republic
CB	*Coxiella burnetii*
CBA/J	Mouse strain
CCHF	Congo-Crimean hemorrhagic fever
cDNA	Complementary deoxyribonucleic acid

CFU	Colony-forming units
cGMP	Cyclic guanosine monophosphate
CHG	Chagres virus
CHIK	Chikungunya virus
CHO	Chinese hamster ovary
CK	Cholecystokinin
CM	Chloroform-methanol
CME	Chloroform-methanol extract
CMR	Chloroform-methanol residue
CNS	Central nervous system
Con-A	Concanavalin A
CRM	Cross-reacting material
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocytes
3,4-DAP	3,4-Diaminopyridine
DA	Department of the Army
DANS	4-Deacetyl-neosolaniol
DAS	Diacetoxyscirpenol
DBA	Mouse strain
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOD	Department of Defense
DON	Deoxynivalenol
DTT	Dithiothreitol
DXM	Dexamethasone
EA	Extractable antigen
EBO	Ebola virus

ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetate
EF	Edema factor
EHF	Ebola hemorrhagic fever
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EP	Embryonated egg passage
ETOH	Ethanol
FA	Fluorescent antibody
FAB	Fast atom bombardment
FASEB	Federation for the Society of Experimental Biology and Medicine
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDP	Filbrin degradation products
FPL	Leukotriene antagonist
G1,G2	Glycoproteins (viral)
GLP	Good Laboratory Practice
GP	Guinea Pig
HBSS	Hank's balanced salt solution
HEV	High endothelial venules
HFRS	Hemorrhagic fever with renal syndrome
HI	Hemagglutination inhibition
HMC	Hubei Medical College
HP	Human passage
HPLC	High-pressure liquid chromatography
HPLC-MS	High-pressure liquid chromatography/mass spectrometry
HT	Dihydroxy T-2 toxin
HTLV	Human T-lymphotrophic virus (AIDS)

HV (HTN)	Hantaan virus
ICR	Mouse strain
ID_{50}	Median infective dose
IDC	Interdigitating dendritic cells
IFA	Indirect immunofluorescence assay
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
ILIR	In-house laboratory research
INF	Infected
IND	Investigational new drug
INT-2	Interleukin-2
JEV	Japanese encephalitis virus
JOS	Josiah strain of Lassa fever virus
JV	Junin virus
KAV	Strain of *Coxiella burnetii*
KHF	Korean hemorrhagic fever
KHL	Keyhole limpet hemocyanin
LCM	Lymphocytic choriomeningitis virus
LD_{50}	Median lethal dose
LD_{99}	99% Lethal dose
LDH	Lactose dehydrogenase
LF	Lethal factor
LNI	Neutralizing antibody
LPS	Lipopolysaccharide
LTE_4	Sulfidopeptide leukotrienes
MAB	Monoclonal antibody

MAS	15-Monacetyl-diacetoxyscirpenol
MBG	Marburg virus
MCab	Monoclonal antibody
MDPH	Michigan Department of Public Health
MLV	Multilamellar vesicles
MMD	Minimum mean diameter
MP	Mouse passage
MRDC	Medical Research and Development Command
MRNA	Messenger ribonucleic acid
MRVS	Medical Research Volunteer Subject
MS	Mass spectrometry
MTC	Minimum toxic concentration
MTD	Mean time to death
NC	Nucleocapsid
NIH	National Institutes of Health
NK	Natural killer (cells)
N-M	Neuromuscular (block)
NRC	National Research Council
PBMC	Peripheral blood mononuclear cells
PFU	Plaque-forming units
PGE_2	Prostaglandin E_2
PH	Prospect Hill virus
PHA	Phytohemagglutinin
PI	Phosphotidylinositol
PIC	Pichinde virus
PLAS	Plasmid
PMN	Polymorphonuclear leukocytes
PRNT(PRN)	Plaque-reduction neutralization

PWM	Pokeweed mitogen
QAE	QAE chromatography
RBC	Red blood cells
RFLP	Restriction fragment polymorphism
RIA	Radioimmune assay
RNA	Ribonuclcic acid
RNP	Ribonuclear particle
RFP	Request for proposals
RSV	Respiratory syncytial virus
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Sandfly fever virus
SFF	Sandfly fever
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic-pyruvic transaminase
STI	Sterne-type strain of anthrax
STX	Saxitoxin
TC	Tissue culture
TCA	Trichloroacetic acid
TCID	Tissue culture infectious dose
TP	Tick passage
TSI	The Salk Institute
Ttx	Tetanus toxin
UNINF	Uninfected
USAMRDC	United States Army Medical Research and Development Command
USAMRIID	United States Medical Research Institute of Infectious Diseases
USUHS	Uniformed Services University of the Health Sciences

VEE	Venezuelan Equine Encephalomyelitis Virus
VERO	African green monkey kidney (cells)
VRV-5	M-segment RNA of Rift Valley fever virus
WC	Whol cell
WEE	Western equine encephalitis virus
WN	West Nile virus
WRAIR	Walter Reed Army Institute of Research
WRAMC	Walter Reed Army Medical Center
XJ	Mouse strain
YF	Yellow fever

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